On The Role of Over-Activation of Innate Immunity During Bacterial Infections

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This work is dedicated to my wife Susanne who
is with me with love and supports me greatly in all my enterprises.

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1. Summary

Background: The investigation of antibacterial responses is one of the oldest and most imporant questions in immunology. Since Metchnikoff, phagocytes were pinpointed to be responsible for defense against invading bacterial pathogens by engulfment and digestion. However, although much information exists on specific molecular mechanisms on the single cell level, phagocyte kinetics in the organs or their origin and destination during bacterial infections remain largely uncharacterized, although they are probably essential to understand the respective immune responses. We have therefore characterized kinetics of granulocytes during and after bacterial and viral infections and after stimulation with Toll like receptor ligands.

Methods: For characterization and enumeration of granulocytes, we used FACS technology with peripheral blood and bone marrow samples. To further investigate fate of granulocytes in tissues, immunohistochemistry was used. When necessary, bacterial counts were measured by serial plating of organ homogenates on agar. Cytokines were measured by ELISA technology.

Results: The first project “Exhaustion of bone marrow granulocyte numbers during uncontrolled bacterial infections” used a systemic bacterial infection and revealed a reduction of viable granulocytes in the bone marrow that correlated directly with the bacterial load in infected spleen and liver (Manuscript in preparation).

In the second part of this work “Increased susceptibility to bacterial superinfection as a consequence of innate antiviral responses”, a virus infection was given prior to bacterial superinfection. Virus infection led to apoptosis and decreased numbers of granulocytes in the bone marrow by interferon type I induction. During this granulocytopenia, bacterial superinfection was greatly facilitated. This paper has been published in *Proc Natl Acad Sci* U S A. 2006 Oct 17;103(42):15535-9. Epub 2006 Oct 9.

In a third project “Disruption of the bone marrow granulocyte supply and uncontrolled bacterial proliferation by TLR2 activation”, an innocuous bacterial infection was given and systemic stimulation of TLR2 performed. Thereafter, bone marrow granulocytes underwent apoptosis and the bacterial infection became uncontrollable (Manuscript in preparation).

In the fourth part, we stimulated the innate immune system with lipopolysaccharide and determined whether lasting changes occurred. We found that a prolonged innate reactivity resulted that led to increased anti-bacterial resistance and survival. This work is currently still in progress.

Conclusion: The cellular kinetics of granulocytes were found to be volatile and crucial for the outcome of bacterial infection. The observed activation-associated apoptosis of bone marrow granulocytes by interferon and toll like receptors could be of some importance in survival of infections and probably should be investigated closer in humans.

2. Abbreviations:

Ab	Antibody
APAAP	Alkaline phosphatase
AS	Aminoacid
BSA	Bovine Serum Albumine
C. elegans	Caenorhabditis elegans
CD	Cluster Designation
CD115	M-CSF receptor, expressed on monocytes
CD11b	ß2-integrin on the surface of macrophages, monocytes and granulocytes
$CD4^+$	CD4 antigen positive cells, T helper cells
$CD8^+$	CD8 antigen positive cells, cytotoxic T cells
Cfu	Colony forming units (for bacterial titers)
DNA	Desoxyribonucleinacid
Drosophila	Drosophila melanogaster
ECACC	European Collection of Animal Culture Cells, Salisbury, UK
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FSL-1	Ligand based on the Mycoplasma salivarium lipoprotein with the formula S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/monocyte-colony stimulating factor
GR-1	Granulocyte antigen 1
HRP	Horse Radish Peroxidase
IFN	Interferon
IFN type I	IFN α/β
IFN type II	IFN γ
Ig	Immunglobulin
IHC	Immunohistochemistry
IL	Interleukin
L.m.	Listeria monocytogenes
LCMV	Lymphocytic choriomenigitis virus
LPS	Lipopolysaccharide, TLR4 ligand

Ly-6C	Surface antigen specific for murine monocytes (high expressors) and granulocytes (intermediate expressors)
Ly-6G	Surface antigen specific for murine granulocytes
M.	Morbus
M-CSF	Multi-colony stimulating factor
Pam2Cys	Lipopeptide consisting of Palmitoyl side chains acid and a cysteine: S-(2,3-bis(palmitoyloxy)propyl)cysteine, TLR2/6 ligand
Pfu	Plaque forming units (for virus titers)
Poly(I:C)	Double strand RNA consisting of Inosins and Cytosins
RNA	Ribonucleinacid
Rpm	Rounds per minute
S. aureus	Staphylococcus aureus
S. typhimurium	Salmonella typhimurium
SIRS	Systemic inflammatory response syndrome
TLR	Toll like receptor
TLR 2/6	TLR2 dimerizes with TLR6, binds bacterial and fungal lipopeptides
TLR3	TLR3 binds double-stranded RNA, i.e. Poly(I:C)
TLR4	TLR4 binds lipopolysaccharide
TNF	Tumor necrosis factor
VSV	Vesicular stomatitis virus
VRSA	Vancomycin resistant staphylococcus aureus

3. Introduction

3.1 Evolution of infections and early defense mechanisms

Parasitic-host relationships have developed between organisms early in evolution of biological systems (Remy, Taylor et al. 1994). Resisting invasion and digestion by other life forms is a crucial factor for evolutionary success in all species, from molluscs to homo sapiens (Boots and Bowers 1999; Ellis, Dodds et al. 2000; Boots and Bowers 2004). Organisms engaging in infectious behaviour have been identified starting from viruses, the lowest known life forms, to highly evolved multicellular eukaryontic organisms. The most common and dangerous infections in humans are by bacteria, which have represented a great scourge and were very difficult to cure until the discovery of penicillium by Alexander Fleming in 1928. Before this time, treatment consisted mainly in administration of carbolic acid (Cheyne 1895), iodine, bismuth salts, zinc compounds, formaldehyde and heat (Pilcher 1899). For over 50 years now, antibiotics have greatly reduced the number of deaths due to bacterial infections. However, many of the common bacterial strains that were feared before penicillium are now slowly acquiring resistance genes to many antibiotics (Chang, Sievert et al. 2003; Whitener, Park et al. 2004). Due to this increasing problem, new interest has arosen to understand and influence the immunological events occuring in anti-bacterial responses.

The archetypical defense weapon against parasitic invasion by other organisms is the phagocytic cell (Metchnikoff 1905). Even a rather primitive organism, the nematode C. elegans that is lacking blood circulation and immune cells, has phagocytotic, although not specialized cells (Kinchen, Cabello et al. 2005). The fruit fly Drosophila melanogaster harbors so-called specialized plasmatocytes, which have high phagocytic activity (Franc 2002; Meister 2004). Phagocytes lack adaptable antigen receptors and have to rely on pattern recognition. Invariable receptors recognise molecular patterns of invading organisms and lead to phagocyte activation. This results in phagocytosis of the respective antigen source and digestion. The concept of pattern recognition receptors has remained conserved in the evolutionary ladder from C. elegans (Hoffmann, Kafatos et al. 1999; Liberati, Fitzgerald et al. 2004) up to homo sapiens. The most prominent among them are the so-called Toll like receptors (Takeda, Kaisho et al. 2003). The recognised pattern is usually a class of molecules that is not present in the respective organism, but is expressed by

invading organisms, such as lipopolysaccharide in gram-negative bacteria. With this simple non-self recognition, phagocyte activation can take place rapidly.

In higher organisms, the adaptive immune system developed with antibody production and MHC I-restricted cytolysis and inflammatory cytokine production. At first, the adaptive immune cells had potent phagocytic abilities (Li, Barreda et al. 2006) that are, however, lost in higher vertebrates. While the actual phagocytosis is performed by granulocytes, macrophages and dendritic cells, it has been shown that primed T cells produce inflammatory cytokines upon bacterial challenge that activate macrophages. However, for the antibacterial response, the presence of phagocytes is a condition sine qua non (Rakhmilevich 1995).

In human and mouse, two broad classes of specialized phagocytes have been described, the granulocyte and the macrophage. In the following section, known facts about granulocytes and macrophage immune function are summarized.

3.2 Granulocytes

In the light microscope, granulocytes are identifiable by their polymorphic nucleus and the presence of typical granula in the cytoplasm. Their name is derived from Paul Ehrlich, who first described them and the nature of the granula within (Ehrlich 1900). At the timepoint of their discovery, the staining technique allowed to differentiate granula by dye uptake. The mature granulocyte harbors several antibacterial agents in two types of granula, the azurophil and specific granula (Borregaard and Cowland 1997). Azurophil granula contain the myeloperoxidase that is used to produce hydrogen peroxide H_2O_2. This substance has a strong antibacterial effect. Elastase, defensin and lysozyme are also found within these granula. Elastase can degrade elastic fibers and outer membrane proteins from Shigella and Escherichia coli (Belaaouaj, Kim et al. 2000). Defensin (Ganz 1999) peptides are highly active against many bacteria. Lysozyme (Ellison and Giehl 1991) is the classic proteolytic enzyme that is also used in lysosomes and in saliva to degrade foreign as well as native proteins. So-called specific granula contain gelatinase, presumably for dissolving basement membranes (Borregaard and Cowland 1997), and the protein lactoferrin of still unknown function. Upon activation of the granulocyte, all these mediators can be released in the process of degranulation.

Granulocytes are short-lived cells that are produced in the bone marrow in quantities comparable to erythrocytes upon stimulation of granulopoiesis by the cytokine G-CSF (Babior 2001). Their half-life, however, is only a tenth of the erythrocyte: About 2-10 days (Babior 2001). After maturation, at least a part of the granulocytes leaves the bone marrow and enters the blood stream (Martin, Burdon et al. 2003). In humans, they constitute about 70% of all blood leukocytes, in mice about 10%. From there, granulocytes can infiltrate peripheral tissues in a cytokine- and integrin-dependent process (Parkos, Delp et al. 1991; Broaddus, Boylan et al. 1994; Furie and Randolph 1995; Parkos 1997) or re-enter the bone marrow in a CXCR2 and CXCR4-dependent process (Martin, Burdon et al. 2003). Only very few granulocytes are found in peripheral tissues under normal conditions except in the spleen, where aged granulocytes are thought to be cleared from the blood stream by macrophages (Suratt, Young et al. 2001). In the mouse, also a small part of granulopoiesis has been shown to occur in the spleen (Golde, Faille et al. 1976). When a peripheral bacterial infection occurs, a large number of granulocytes quickly accumulates at the bacterial lesion beginning a few hours after infection (Mackaness 1962). These granulocytes fulfill their anti-bacterial function and are degraded to cellular debris (*pus*) that form the center of the newly formed abscess. In this manner, a large number of granulocytes can be consumed. Cellular debris is then cleared by infiltrating macrophages. It has been found that administration of LPS as an inflammatory stimulus results in heightened granulopoiesis (MacVittie and Walker 1978; Levitt and Quesenberry 1982). The survival time of a granulocyte after entering tissues is largely unclear. One study showed with peripheral blood granulocytes that their survival time in vitro can be prolonged by administration of acute phase cytokines such as IL-1a, IL-6 and TNF-α (Colotta, Re et al. 1992). Other studies have shown that granulocyte survival is shortened when anti-bacterial mechanisms are activated such as the NADPH Oxidase enzyme (Lundqvist-Gustafsson and Bengtsson 1999). We have investigated this question also to some extent (see below).

When peripheral blood, splenocytes or bone marrow are analysed by FACS technology, granulocytes can be easily identified by their light scattering properties and size. Apart from morphology, granulocytes can be specifically stained with monoclonal antibodies, which we have always done for the experiments in these studies. By immunohistochemistry or FACS, the surface antigens Ly6G, which

overlaps with the GR-1 antigen, and the activation-induced β2 integrin CD11b are usually used for identification of granulocytes in the bone marrow as well as in the periphery (Fleming, Fleming et al. 1993).

Granulocytes have different states of activation (Hallett and Lloyds 1995). In the bone marrow, their anti-bacterial mechanisms are usually dormant, which is presumably important as great immunopathology can occur when granulocytic enzymes are set free inappropriately (Schmitz, Kurrer et al. 2005). One example of a pathological granulocyte-dependent immunopathology is Wegener's disease where antibody-dependent lysis of peripheral granulocytes occurs, leading to inflammation and formation of granulomas (van Rossum, Limburg et al. 2005). Several methods have been described in the literature that give information about the activation status of granulocytes. Upregulation of the β2 integrin CD11b on the surface of granulocytes can be measured as an early marker during activation (Kuijpers, Tool et al. 1991) by FACS, usually as mean fluorescence intensity. Another indication of activation is the NADPH oxidase activity (Benna, Dang et al. 1997). This enzyme is involved in production of superoxide (O_2^-) and is the key player of most antibacterial effector functions (Reeves, Lu et al. 2002). Its activity can be measured by oxidation-activated fluorescent dye like DHR123 (Smith and Weidemann 1993). H_2O_2, an antibacterial agent of lesser importance that is produced by activated myeloperoxidase, can also be measured in a similar manner. Degranulation of anti-bacterial enzymes is measurable by specific ELISA and has been established for β-lactoferrin. An assay that is less often used nowadays is oxygen consumption by activated granulocytes (Proctor 1979).

In clinical medicine, lack of peripheral or bone marrow granulocytes signifies a high risk of bacterial or fungal infections. For experimental induction of agranulocytosis, granulocytes can be depleted by administration of monoclonal antibody against surface antigens (GR-1) (Czuprynski, Brown et al. 1994). How the depletion works is not elucidated, but as few as 25μg of the antibody are sufficient to eliminate >98% of granulocytes in the blood, spleen and bone marrow (Tvinnereim, Hamilton et al. 2004) for at least 3 days (own observation).

3.3 Macrophages and Monocytes

Macrophages are characterized by relatively large sizes of >12μm and a large non-convoluted nucleus (Wiktor-Jedrzejczak, Ansari et al. 1992). Often, they have a foamy pattern in the cytoplasm, which is associated with phagocytic activity. Macrophages are found everywhere in the body, and both motile and immotile forms exist – however, unlike granulocytes, they are often adapted to the organ microarchitecture and have been given specific names depending on their localization (Chan, Leenen et al. 1998; Ziegler-Heitbrock 2000). Free and motile macrophages are found in the peritoneum and in the blood. On the other hand, fixed macrophages are found in solid organs such as the Kupffer cells in the liver, lining the capillaries (Pulford and Souhami 1981), red pulp macrophages (Martinez-Pomares, Hanitsch et al. 2005), marginal zone (Kraal and Janse 1986) and follicular tingible body macrophages in the spleen (Mueller, Cremer et al. 2001), alveolar macrophages in the lung and so forth. Especially the Kupffer cells have been reported to be immotile although they can protrude into lumen of neighbouring capillaries with ameboid movements (Motta 1975). It has been reported that macrophages can divide in tissues, but they originally arise from the bone marrow with a turnover of several weeks after irradiation (Gale, Sparkes et al. 1978).

Macrophages were first identified by Metchnikoff as having phagocytotic activity during infections (Metchnikoff 1905). During bacterial infection with Listeria monocytogenes, has been observed that infiltrating mononuclear cells replace granulocytes starting from day 3 to 4 (Mackaness 1962). These cells arise from the blood (North 1970; Moghimi 2002) or from dividing fixed tissue-resident macrophages such as Kupffer cells (North 1969). They have been identified to clear debris (Krysko, Denecker et al. 2006), have bactericidal activity (Mackaness 1962); (Leist, Heuchel et al. 1988) and present antigens on MHC class II (Janeway 1989).

During bacterial infection with Listeria monocytogenes, resident macrophages of the spleen and liver are the first cells to harbor bacteria (Cousens and Wing 2000). They play a crucial role in reducing the bacterial load.

To deplete macrophages for experiments, it is possible to administer liposomes loaded with the agent clodronate (van Rooijen and van Nieuwmegen 1984). These liposomes are actively phagocytosed and clodronate causes macrophages to enter apoptosis. This method is greatly effective for Kupffer cells and fixed splenic

macrophages. After depletion of these macrophages by clodronate, the susceptibility to L.m. is greatly increased (Ebe, Hasegawa et al. 1999).

At least some macrophages arise from infiltrating monocytes. They are characterized by a relatively large size with 12-15μm, a large pale and bean-shaped nucleus and relatively clear cytoplasm. These cells arise from the bone marrow during monocytopoiesis which can be stimulated by administration of M-CSF and GM-CSF (Merchav and Wagemaker 1984). Blood monocytes can be measured in FACS by the surface antigens CD11b and high expression of the M-CSF receptor CD115 (Sunderkotter, Nikolic et al. 2004) or high expression of the surface antigen Ly6C (Serbina and Pamer 2006), whereas granulocytes express only intermediate amounts of Ly6C. Bone marrow monocytes and their precursors have not been clearly identified by FACS surface markers, although some have used the same surface antigens as in blood. It has been shown that upon inflammatory stimulation of the peritoneum, infiltrating macrophages arise from the bone marrow monocytes. In contrast to this finding, peritoneal macrophages in the normal state have a slow turnover of several weeks (van Furth and Cohn 1968).

Monocytes have been implicated in phagocytosis of pathogens and debris. During *Listeria monocytogenes* infection of the mouse, at day 3 to 4 infiltration of mononuclear cells that are assumed to stem from monocytes are observed at the site of bacterial lesions and replace granulocytes (Mackaness 1962). To gain more insight in the role of the monocytes in actual pathogen clearance, it would be interesting to know whether the abscesses at the transition from granulocytic to monocytic infiltration are still bearing a heavy bacterial load or whether only quasi-sterile abscesses are infiltrated by monocytes for clearance of cellular debris. Interestingly, several groups have made the finding that inflammatory peritoneal macrophages cannot effectively clear bacteria in contrast to resident peritoneal macrophages (Baker and Campbell 1980; Harrington-Fowler, Henson et al. 1981; Sich, Bubel et al. 1987), although their phagocytotic capacity is not impaired.

Monocytopoiesis has been reported to be sensitive to the cytostatic drug etoposide. With this reagent, one could gain information about the role of monocytes during L.m. infection.

3.4 Listeria monocytogenes

We have used Listeria monocytogenes infections in mice for our experiments. This model infection has been used for over 50 years in immunology and much information about the elicited early and adaptive immune responses is available. L.m. has originally been described in 1926 as the cause of a lethal disease in a rabbit colony in Cambridge, England (Murray 1926). These rabbits were found to form a great monocytosis in blood, and therefore the bacterium was named *Bacterium monocytogenes*. A nearly concurrent report from South Africa described a grampositive bacterium as the cause of Tiger River Disease, a deadly malady of wild gerbils (Pirie 1927). This disease was characterized by a necrotizing hepatic infection and was named *Listerella hepatolytica* in honor of the british surgeon Joseph Lister. As the bacterium was later found to be identical, the name was changed to *Listeria monocytogenes*. Its natural habitat was found to be soil, with a predilection to grow in dairy products and raw meats from which oral infection results.

L. monocytogenes was subsequently found to cause human disease in pregnant women, leading to septic abortions, and meningitis and sepsis in elderly and immunocompromised individuals (Gellin and Broome 1989).

A large range of mammals have been found to be susceptible to L. monocytogenes, among them the mouse. In the tested laboratory strains, the predominant pathology found is usually the hepatic infection after 2-6 days (Wilder and Sword 1967). After this phase is survived, a later infection of the CNS resulting in meningitis sometimes occurs at 7-21 days (Prats, Briones et al. 1992).

The natural route of infection with L. monocytogenes is the oral ingestion, which results in adherence to intestinal epithelial cells by the bacterium. To achieve this, the protein internalin A is required, which is expressed at the listerial surface and interacts with epithelial cadherin (E-cadherin) at the surface of epithelial cells (Gaillard, Berche et al. 1991). Mice have a point-mutation in the E-cadherin sequence which renders the described interaction much less efficient than in the human intestine, resulting in a relative resistance of mice to oral infection (Lecuit, Vandormael-Pournin et al. 2001; Lecuit 2005). After invasion of the epithelial layer, bacteria enter the blood stream and are internalized by hepatic and splenic tissue macrophages and hepatocytes which offer the hepatocyte growth factor receptor for interaction with listerial internalin B (Shen, Naujokas et al. 2000).

After phagocytosis, L.m. secretes listeriolysin O, a protein which lyses the phagosomal membrane and allows bacteria to escape in the cytosol (Bielecki, Youngman et al. 1990). From this compartment, bacteria start to express the actin-assembly-inducing protein (ActA) at one pole, a bacterial protein that nucleates actin (Domann, Wehland et al. 1992; Kocks, Gouin et al. 1992), creating actin-polymers that propel the bacteria through the cytoplasm into neighbouring cells. This mode of propagation is essential for listerial virulence as knockout variants of the bacterium are strongly attenuated (Goossens and Milon 1992).

Although the natural route of infection is oral, most laboratory studies use the intravenous or intraperitoneal route, thereby focusing on the systemic phase of the infection and disregarding the mucosal as well as the mucosal-induced systemic immune response.

Early during infection, an effective acute phase response is essential for limiting the infection. TNF-α as well as IFN-γ are required, as demonstrated with knockout mouse studies (Buchmeier and Schreiber 1985; Havell 1989; Pfeffer, Matsuyama et al. 1993). These cytokines are presumably activating tissue macrophages that have ingested L.m. to upregulate lysosomal processing and limiting phagosomal escape. Other cytokines such as IFN-α have been found to be detrimental for the anti-listerial innate immune response (Auerbuch, Brockstedt et al. 2004; Carrero, Calderon et al. 2004; O'Connell, Saha et al. 2004). Bacteria are destroyed by infiltrating phagocytes, among them first the granulocytes (Rakhmilevich 1995) and then later the macrophages (Mackaness 1962). The granulocytes reduce an initial inoculum of L.m. that reaches mainly the liver by more than 90% within 12 hours (Conlan and North 1994). It has been shown that the number of bone marrow granulocyte/macrophage colony precursors correlates positively with the innate resistance to L.m. (Young and Cheers 1986). In the first 3 days, the bacteria are held in check by these two types of phagocytes. Later, T cells (Zinkernagel, Blanden et al. 1974) are primed and infiltrate the infected organs. Their role is mainly to produce inflammatory cytokines such as IFN-γ for activation of phagocyte killing activity (Leist, Heuchel et al. 1988; Leist, Meager et al. 1991), as direct lysis of infected cells has not been demonstrated. There is a high-titered antibody response against listeria monocytogenes, but it is not protective (Miki and Mackaness 1964).

3.5 Staphylococcus aureus and Salmonella typhimurium

As further infection models, we used Staphylococcus aureus and Salmonella typhimurium. S. aureus is a grampositive coccus that grows in clumps and is considered one of the major human pathogens (Kowalski, Berbari et al. 2005). It has a great predilection for causing purulent infections of the bones (osteomyelitis), heart valves (endocarditis) and often, localized infection results in sepsis. Unfortunately, there are S. aureus strains that have acquired resistance genes to all known antibiotics including vancomycin (VRSA) (Chang, Sievert et al. 2003). Although these infections can still be treated by elevation of the vancomycin concentration close to the threshold of toxicity for the patient in combination with oxacillin, the prognosis is that sooner or later, these strains might not be cureable anymore with antibiotics.

In the mouse, S. aureus has been used as a model infection. It produces septic disease early after infection (Verdrengh and Tarkowski 1997). If low inoculation dosages are used, chronic purulent infection of the articulations develop. S. aureus has been reported to be phagocytosed by granulocytes and macrophages (Kowalska, Szydlowska et al. 1987; Kielian, Barry et al. 2001), and with depletion studies it has been shown that both cellular subsets are essential, as with L.m. (Verdrengh and Tarkowski 1997; Tanaka, Miyazaki et al. 2004). However, T cells have not been shown to play a role for clearance of this pathogen.

Salmonella typhimurium is a gramnegative rod that is present in asymptomatic carrier patients (Bhan, Bahl et al. 2005). The infection is acquired by ingestion of contaminated foods and results in gastrointestinal symptoms such as diarrhea, vomiting, fever and malaise. In the mouse, it is used as oral or i.v. infection and results in infection of solid organs (Lin, Wang et al. 1987; Wang, Lin et al. 1988) where the bacteria are destroyed within neutrophil granulocytes and macrophages (Gellin and Broome 1989). As with L.m., it has been shown that correlations exist between granulocyte bactericidal activity and susceptibility of the respective mouse strain to Salmonella typhimurium (van Dissel, Stikkelbroeck et al. 1986).

3.6 Lymphocytic choriomeningitis virus

LCMV is an enveloped, single stranded RNA with two segments (Buchmeier 2001). It mainly infects macrophages and dendritic cells, but can be pleiotropic as soon as infection is established. LCMV induces a strong response by innate immunity that is characterized by high secretion of IFN I, produced mainly by dendritic cells (Dalod, Salazar-Mather et al. 2002). After day 4, it induces a cytotoxic $CD8^+$ T cell response that clears the virus. We have used LCMV mainly as an inducer of IFN I.

With these model infections, we have addressed several questions which are detailed in the introductions of the different chapters. In the following studies, we have reproduced some classical findings and found an important role for the kinetics of granulocytes during the whole period of L.m. infection. We have investigated the course of infection when granulocytes are systemically activated by either virus-induced type I interferon, or by ligation of toll like receptor 2 with Pam2Cys, simulating a grampositive sepsis. In another approach, we stimulated the innate immune system with lipopolysaccharide and detected a prolonged reactivity that resulted in heightened anti-bacterial resistance.

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4. Aim of the presented research work

4.1 General considerations

The bone marrow as the origin of circulating granulocytes has long been neglected in analysis during infections. A striking lack of reports of the interplay between GRC infiltration of bacterial lesions and GRC reserve in the bone marrow exists. Because the number of GRC present in the blood is of highest medical relevance in all immunocompromised patients, we have started our experiments in analysing this interplay during bacterial and viral infections.

4.2 Bone marrow granulocyte reserve during bacterial infections

In the course of our experiments, we found that the number of bone marrow granulocyte is affected during bacterial infections. We therefore strove to investigate the relation between bacterial load and granulocyte reserve.

4.3 Bone marrow granulocyte reserve during viral infections

When the LCMV infection was analysed for influence on the granulocyte reserve, increased apoptosis and lowered numbers of granulocytes was found in a time window early after the infection. In further experiments, it was then analysed whether this influenced the susceptibility to bacterial super-infection.

4.4 Bone marrow granulocyte reserve after systemic TLR2 ligation

By administration of TLR2 ligands, it was found that granulocytes reacted immediately with great activation and heightened cell death in the bone marrow. We used this effect to investigate the influence of a sudden increase of circulating bacterial products on the resistance to a non-lethal bacterial infection.

4.5 Prolonged innate reactivity

When later timepoints after administration of TLR ligands, bacterial or viral infections were analysed, a consistent elevation of bone marrow granulocytes was found. Further we showed that after a sudden reduction of their number, bone marrow granulocytes accumulate in elevated numbers for a certain time. We then analysed whether this corresponded to a simple form of "memory" that would enable the preexposed mouse to react more strongly to a renewed stimulus.

5. Exhaustion of bone marrow granulocyte numbers during uncontrolled bacterial infections

5.1 Abstract

Bacterial infections capable of infesting tissues require phagocytes for clearance. We have used the model system Listeria monocytogenes to study phagocyte responses to bacterial infection. We observed that during the whole period of active infection, granulocytes were recruited to bacterial lesions. Granulocyte numbers in the bone marrow were reduced accordingly, correlating with the number of bacteria inoculated. Blood granulocyte numbers increased but reached a plateau independent of the inoculum. We further observed that during overwhelming infection, all bone marrow granulocytes could be depleted until supply of fresh granulocytes to abscesses ended and insufficient bacterial containment occurred. This led to sepsis and death.

5.2 Introduction

Granulocytes are the most short-lived cells of the body. Their main function is to actively combat invading pathogens by phagocytosis, oxidative agents and enzymatic digestion. Granulocytes have their origin in the bone marrow where granulopoiesis occurs (Babior 2001). This is a process of development from stem cells that takes about 14 days. It is thought that it can be shortened upon heightened demand of granulocytes in the periphery. After maturation, granulocytes are kept in the bone marrow for a few days until release in to the blood stream. Only a small part of the available granulocytes are circulating at a time, about 1 million in the blood of the mouse compared to 64 million granulocytes in the whole bone marrow (Babior 2001; Blankenberg, Tait et al. 2001). Circulation time is measured in hours and ends in marginalization and diapedesis through capillary walls (Babior 2001). After entering peripheral tissues, granulocytes use chemotaxis to approach inflammatory targets (Godaly, Bergsten et al. 2001; Fierer, Swancutt et al. 2002). It is known from in vitro experiments that they can survive up to 3 days when stimulated to some extent with inflammatory acute phase cytokines such as IL-6 or IL-1 (Colotta, Re et al. 1992). However, after full activation, the respiratory burst mechanism is upregulated. Great O_2 consumption is found in activated granulocytes which produce H_2O_2 and O_2- that are used to kill pathogens. This induced oxidative environment is also harmful to the granulocyte which undergoes apoptosis after dispensing these substances (Watson, Redmond et al. 1996; Yamamoto, Taniuchi et al. 2002; Zhang, Hirahashi et al. 2003). The substance *pus* that is found in many places of granulocyte activation is formed primarily of dead granulocytes, cellular detritus and bacteria. *Pus* is later cleared by macrophages and the normal tissue architecture is restored (Hart, Dougherty et al. 1997; Blankenberg, Tait et al. 2001).

Upon inflammatory stimuli, granulocyte numbers in peripheral blood can rise rapidly. However, it has been observed that during overwhelming sepsis with organ dysfunction and blood pressure insufficiency, granulocyte numbers are either very high or very low. Both >12000 / ul and <4000 / ul leukocytes in peripheral blood have been shown to contribute to the SIRS syndrome that carries a poor prognosis for the patient (Muckart and Bhagwanjee 1997). Granulocyte release from the bone marrow is known to depend on inflammatory stimuli (Burdon, Martin et al. 2005).

We have asked whether increased bacterial numbers also results in heightened granulocyte release from the bone marrow, and whether this would be reflected in blood granulocyte numbers as a transitory state.

As it is as yet unclear if and to what extent granulocyte activation is needed for release from the bone marrow, we measured CD11b upregulation in the bone marrow as a marker for activation.

Granulocyte activation is a two-edged sword and can lead to enhanced survival (Colotta, Re et al. 1992) as well as apoptosis (Zhang, Hirahashi et al. 2003). Therefore, we also measured induction of granulocyte apoptosis in the bone marrow.

Further, we asked whether the G-CSF response after emigration of granulocytes from the bone marrow corresponds to the required supply. This question is of interest as administration of recombinant G-CSF has been shown to be beneficial in sepsis, implying insufficient production of G-CSF during bacterial infection.

5.3 Material and Methods:

Mice

Specific pathogen free (SPF) laboratory mice were from the Institute of Labortierkunde of the veterinary facility of the University of Zurich. Experiments were performed according to Swiss veterinary law and institutional guidelines. C57BL/6 mice were used.

Infections

Listeria monocytogenes (L.m.) strain 10403S grown overnight in Brain Heart infusion broth, washed two times in PBS and injected i.v. in 200 ul.

Bacterial titer

Bacterial titers were determined in homogenized halfs of spleen, the left lobe of the liver, 100ul of flushed bone marrow or 50ul of peripheral blood in PBS with serial dilutions plated on brain heart infusion or blood agar plates.

Bone marrow aspirates and culture

Bone marrow cells were flushed with PBS from the femur and were stained for FACS analysis or cultured in RPMI with 10% FCS.

FACS and antibodies

Anti-Ly6G, GR1, CD11b, Annexin V and 7-Amino-Actinomycin-D (7-AAD) were obtained from BD, Basel. Cells expressing Ly6G / GR1 and CD11b are termed “GRC”. FSC/SSC gates were used to exclude debris. A fixed number of fluroescent APC beads were used to quantify cell number per sample volume.

Histology

Histological samples were snap-frozen in Hanks medium and stained with an anti-L.m. rabbit serum (a gift from Prof J. Bille, Lausanne), Gr-1 (Pharmingen) or F4/80 (BM8, BMA) antibodies. Staining was developed using a goat anti-rat antibody (Caltag Laboratories) or goat anti-rabbit (Jackson Immuno Research) and an alkaline phosphatase-coupled donkey anti-goat antibody (Jackson Immuno Research) with naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid 2-methoxy anilide) phosphate and new fuchsin as a substrate. The presence of alkaline phosphatase activity yielded a red reaction product. The sections were counterstained with hemalum.

Determination of cytokine production

G-CSF was measured with an ELISA from R&D (Minneapolis, USA).

5.4 Results

To determine peripheral blood granulocyte responses to a low dose ($^1/_{10}$ LD_{50}) infection with L.m., C57BL/6 mice were infected with 10^3 colony forming units (cfu) L.m. intravenously and granulocyte numbers were followed in the blood over time by FACS staining with surface markers Ly6G and CD11b. We found that granulocytes increased until day 5 in the blood up to 2000 / μl, then dropped to normal levels again until day 9 (Fig. 1a). During in- and decrease of granulocyte numbers, great variability was found between different animals. To determine whether this would correspond to the number of granulocytes in the bone marrow, eluates from the femur were counted for $Ly6G^+CD11b^+$ cells at the different timepoints. A numerical decrease of $Ly6G^+CD11b^+$ cells was measured during the L.m. infection of about 60% that was followed by a strong increase of granulocyte numbers during later days of the infection (Fig. 1a). Ly6G-negative $CD11b^+$ monocytic cells (Lagasse and Weissman 1996; Dahl, Walsh et al. 2003) did not show a similar reduction. All mice survived the infection at least 30 days (Fig. 1c).

We then asked whether granulocyte reduction in the bone marrow correlated with the bacterial load at the different timepoints. Therefore, organs were harvested at the different timepoints, homogenized and plated on agar (Fig. 1b). Bacterial titers in organs increased until day 3 up to 5 x 10^6 cfu per organ, then dropped again below the detection limit at day 9. The numerical reduction of granulocytes in the bone marrow correlated directly with the bacterial load in spleen and liver ($p<0.0001$) (Supp. Fig. 1). To determine whether granulocyte consumption was responded to by increased stimulation of granulopoiesis, G-CSF was measured in serum. Levels of G-CSF were highest at day 2 and then decreased to undetectable levels until day 7 (Fig. 1d).

Next, it was investigated whether the decrease of granulocytes in the bone marrow was paralleled with infiltation in infected tissues. At the different timepoints, immunohistology was performed on the liver and spleen with the marker GR-1 (Ly6G/C), and macrophages with the pan-macrophage specific marker F4/80. In the liver, no granulocytes are found in the naïve state. After infection, granulocytic infiltrates were visible starting from day 2 of the infection, forming small abscesses around bacterial lesions, with maximal granulocytic abscesses at day 4. $F4/80^+$ cells infiltrated abscesses at day 3-4 and starting from day 5-7 replaced granulocytes.

However, granulocytes were present during the whole period of the infection at the bacterial lesion (Fig 2a). We found that granulocytes were present already in the naïve state in the red pulp of the spleen. Beginning at day 1 after infection, the number of granulocytes increased and started to concentrate around bacterial lesions. The strongest infiltates were found at day 4 where dense clumps of granulocytes filled about a fifth of the splenic parenchyma. Granulocyte aggregates then started to thin out but persisted around regions of bacterial presence until day 9, when no more bacteria were measurable with titration of the organ homogenate. Macrophages were present in great numbers already in the naïve state and did not visibly change. However, lymphoid follicles were strongly decreased in size at day 5 (Fig. 2b). We also stained for Listeria monocytogenes bacteria by polyclonal antibody, but could not demonstrate bacteria immunochemically during the infection with 10^3 cfu L.m. (Fig. 2a, b).

To analyse the immune response in a situation that could not be easily contained by the immune system, 10^5 cfu L.m. were used for infection. C57BL/6 mice showed a rapid linear decrease of more than 50% of the original granulocyte number at 24 hours in the bone marrow (Fig. 1e), while at 48 hours, only about 10% of the original amount of granulocytes was left in the bone marrow. The Ly6G-negative $CD11b^+$ cells that have been shown to be of the monocytic lines did not decrease during the infection, suggesting that the observed phenomenon was specific for granulocytes (Fig. 1e). Blood granulocytes increased until 24 hours p.inf., then were decreased again at 48 hours p.inf. (Fig. 1e). Interestingly, and contrary to expectation, granulocytes never reached a higher level in the blood than during the 10^3 cfu infection. The bone marrow granulocyte depletion was accompagnied by a strong increase in G-CSF in serum (Fig. 1d). Determination of listerial titers in the spleen and liver showed a rapid increase in titers (Fig. 1f) until day 3 when animals showed clinical signs of disease and were sacrificed. At this timepoint, animals also harboured bacteria in the bone marrow (Fig. 1f). When a linear regression was calculated, we found that the elevation of bacterial titers could significantly predict a lower bone marrow granulocyte number (Supp. Fig. 1).

Histological analysis showed dense infiltrates of granulocytes in both liver and spleen (Fig. 4a, b) as early as day 1. At day 3, abscesses contained dead granulocytes in the center, but at the active edge of the abscesses, no viable granulocytes were present.

This corresponded to propagation of L.m. in hepatocytes at the edge of these insufficient abscesses, visible by strongly stained hepatocytes filled with bacteria. Therefore, exhaustion of bone marrow granulocytes correlated with insufficient bacterial containment in abscesses. In the spleen, the granulocyte infiltrations also lost their cellular integrity at day 3, although no clear abscesses or listerial propagation could be discerned.

To determine whether granulocytes were activated in the bone marrow and blood by the infection, we measured CD11b expression on granulocytes, which has been repeatedly shown to be an activation marker (Kuijpers, Tool et al. 1991). Both inocula led to granulocyte activation in the bone marrow and blood (Fig. 3a,b). The mice that had been infected with 10^3 cfu L.m. showed increased activation in both compartments for several days during the active infection, then went back to the level of the naïve state. In the group with 10^5 cfu L.m., granulocyte activation showed a linear increase during the infection with the highest value at the time of clinical disease. Interestingly, this was not accompagnied by heightened bone marrow macrophage ($CD11b^+Ly6G^-$) activation. There was no significant difference regarding granulocyte CD11b upregulation between the two inocula. However, when the percentage of apoptotic granulocytes was examined at the different timepoints, inoculation with 10^5 cfu L.m. led to >50% apoptotic bone marrow granulocytes already at day 2 and >70% at day 3 (Fig. 3c,d). Infection with 10^3 L.m. instead induced granulocyte apoptosis not more than 12%. In the blood, almost no apoptotic granulocytes were measurable at all times (not shown), possibly due to rapid sequestration by the spleen. Taken together, during L.m. infection, activation was detectable in both blood and bone marrow granulocytes. After inoculation of a high dose of L.m., exhaustion of bone marrow granulocytes was observed, correlating with insufficient clearance and reduction of granulocytic infiltrates in infected organs.

5.5 Discussion

Listeria monocytogenes infection resulted in recruitment of granulocytes to the blood stream and infiltration in spleen and liver. Granulocyte emigration from the bone marrow and infiltration correlated with bacterial load in target organs. G-CSF secretion correlated inversely with the granulocyte reserve in the bone marrow. Activation of granulocytes in the bone marrow and blood was independent of bacterial load in the organs. However, apoptosis induction was found to a strong extent in the bone marrow of high dose challenged animals.

By administrating an overwhelming dose of L.m., we could observe that without exception, as soon as the mouse started showing symptoms of uncontrolled bacterial proliferation and clinical disease, the bone marrow granulocyte reserve was deprived of live granulocytes. Likewise, the bone marrow granulocyte reserve was never depleted when the infection was not life-threatening. When granulocytes were depleted during an infection with 10^3 L.m. with monoclonal antibody NimpR14, bacteria started uncontrolled growth immediately (not shown, see third chapter). Granulocytes have also been found to be essential for containment of L.m. during primary and secondary infection (Rakhmilevich 1995). In addition, it has been shown that tissue macrophages are also necessary for overcoming the L.m. infection (Samsom, Annema et al. 1997). It is less clear what role monocytes have, and no studies with selective monocyte depletion have been performed to date. When in our study, histology of the 10^5 cfu L.m. infection was investigated for $F4/80^+$ cells, we have found only few hepatic and splenic abscess-infiltrating macrophages until day 3 (Fig. 4a). We therefore hesitate to attribute an important role to infiltrating macrophages in failed survival of this high dose of listeria during the days 3. Instead, failed containment of L.m. seems to be at the level of granulocyte insufficiency.

We observed that almost all granulocytes in the bone marrow were used to counteract a peripheral bacterial infection. Furthermore, in the naïve, unprimed state, granulopoiesis was not quick enough to produce enough granulocytes to overwhelm a high number of bacteria. If granulopoiesis were induced by prior G-CSF treatment or inflammatory stimuli, the number of granulocytes produced in emergency situations could be much higher (Noursadeghi, Bickerstaff et al. 2002). This could also be directly correlated to survival. Clinical studies have been performed with GM-CSF treatments or direct granulocyte transfusions. Further, avoiding granulocytopenia during infections is also important during antibiotic therapy as

elevated concentrations of of antibiotics are necessary during granulocytopenia (van der Voet, Mattie et al. 1984).

There have been numerous studies that have used granulocyte transfusions in neutropenic immunosuppressed patients, or in septic patients with neutropenia. Unfortunately, most of these studies showed mixed results. A reason for this could be that transfusion of peripheral blood granulocytes might result in granulocyte populations that are of very limited half-life (4-8 hours as published and corresponding to our own observation, not shown). In addition, peripheral granulocytes probably home much less to the bone marrow when inflammation is already present (Lovas, Knudsen et al. 1996). However, with new in vitro culture techniques, it might be possible to greatly expand bone marrow granulocytes at more immature states. These have been shown to home to the bone marrow and have repopulation potential (Szilvassy, Meyerrose et al. 2001) and could therefore be of much greater value in generating an effective granulocyte reserve that can protect immunosuppressed patients from bacterial infections.

5.6 Author contributions

A. Navarini performed all experiments and wrote the manuscript. Help with experiments as well as intellectual help was provided by K. Lang, M. Recher and A. Verschoor. Histology was performed by Prof. B. Odermatt. The work was performed in the laboratory of Prof. R.M. Zinkernagel and Prof. H. Hengartner who guided the work and manuscript.

5.7 Figure legends:

Figure 1: Infection with 10^3 or 10^5 cfu L.m.

a) $Ly6G^+CD11b^+$ (Granulocytes) and $CD11b^+Ly6G^-$ (Macrophages) cells in BM and $Ly6G^+CD11b^+$ in blood at the respective days after low-dose L.m. infection (10^3 cfu) (n=3-4 per time point, mean +/- SEM, one of two experiments with similar outcome)
b) L.m. titers in spleen, liver, BM (cfu/femur) and blood (cfu/μl) at the respective timepoints (n=4 per timepoint, mean +/- SEM, one of two experiments with similar outcome)
c) Survival of WT mice after infection with low-dose (10^3 cfu) or high-dose (10^5 cfu) L.m. i.v. (n=5-10 per group)
d) Serum concentration of G-CSF after infection low-dose (10^3 cfu) or high-dose (10^5 cfu) L.m. i.v. (n=3-4 per timepoint, mean +/- SEM)
e) $Ly6G^+CD11b^+$ (Granulocytes) and $CD11b^+Ly6G^-$ (Macrophages) cells in BM and $Ly6G^+CD11b^+$ in blood at the respective days after infection with 10^5 cfu L.m. (n=3-4 per timepoint, mean +/- SEM, one of two experiments with similar outcome)
f) Bacterial titers in spleen, liver, BM (cfu/femur) and blood (cfu/μl) at the respective timepoints (n=4 per timepoint, mean +/- SEM, one of two experiments with similar outcome)

Figure 2: Histology timecourse of liver during infection with 10^3 cfu L.m.

a) Livers or b) spleens taken at the respective timepoints were stained immunohistochemically for presence of $GR\text{-}1^+$ or $F4/80^+$ cells and for L.m. bacteria.

Figure 3: Activation and apoptosis during infection with 10^3 or 10^5 cfu L.m.

b) Mean fluorescence intensity of CD11b on the surface of $Ly6G^+CD11b^+$ (GRC) and $CD11b^+$Ly6G- (Macrophages) cells in BM and blood after infection with 10^3 cfu L.m. (n=3-4, mean +/- SEM, one of two experiments with similar outcome)
c) Mean fluorescence intensity of CD11b on the surface of $Ly6G^+CD11b^+$ (GRC) and $CD11b^+$Ly6G- (Macrophages) cells in BM and blood after infection with 10^5 cfu L.m. (n=3-4, mean +/- SEM, one of two experiments with similar outcome)

d) Percentage of Annexin V^+ GRC, macrophages and $Ly6G^-CD11b^-$ cells (Non-phagocytes) in BM after infection with 10^3 cfu L.m. (n=3-4, mean +/- SEM, one of two experiments with similar outcome)
e) Percentage of Annexin V^+ GRC, macrophages and $Ly6G^-CD11b^-$ cells (Non-phagocytes) in BM after infection with 10^5 cfu L.m. (n=3-4, mean +/- SEM, one of two experiments with similar outcome)

Figure 4: Histology timecourse of liver during infection with 10^5 cfu L.m.

a) Livers and b) spleens taken at the respective timepoints were stained immunohistochemically for presence of GR-1^+ or F4/80^+ cells and for L.m. bacteria.

Supplementary Figure 1: Correlation between bacterial load and BM Granulocytes in low and high dose L.m. infection

a) Correlation between the mean of BM granulocytes at the respective days and bacterial titers in liver and spleen after infection with 10^3 cfu L.m.
b) Correlation between the mean of BM granulocytes at the respective days and bacterial titers in liver and spleen after infection with 10^5 cfu L.m.

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Fig. 1

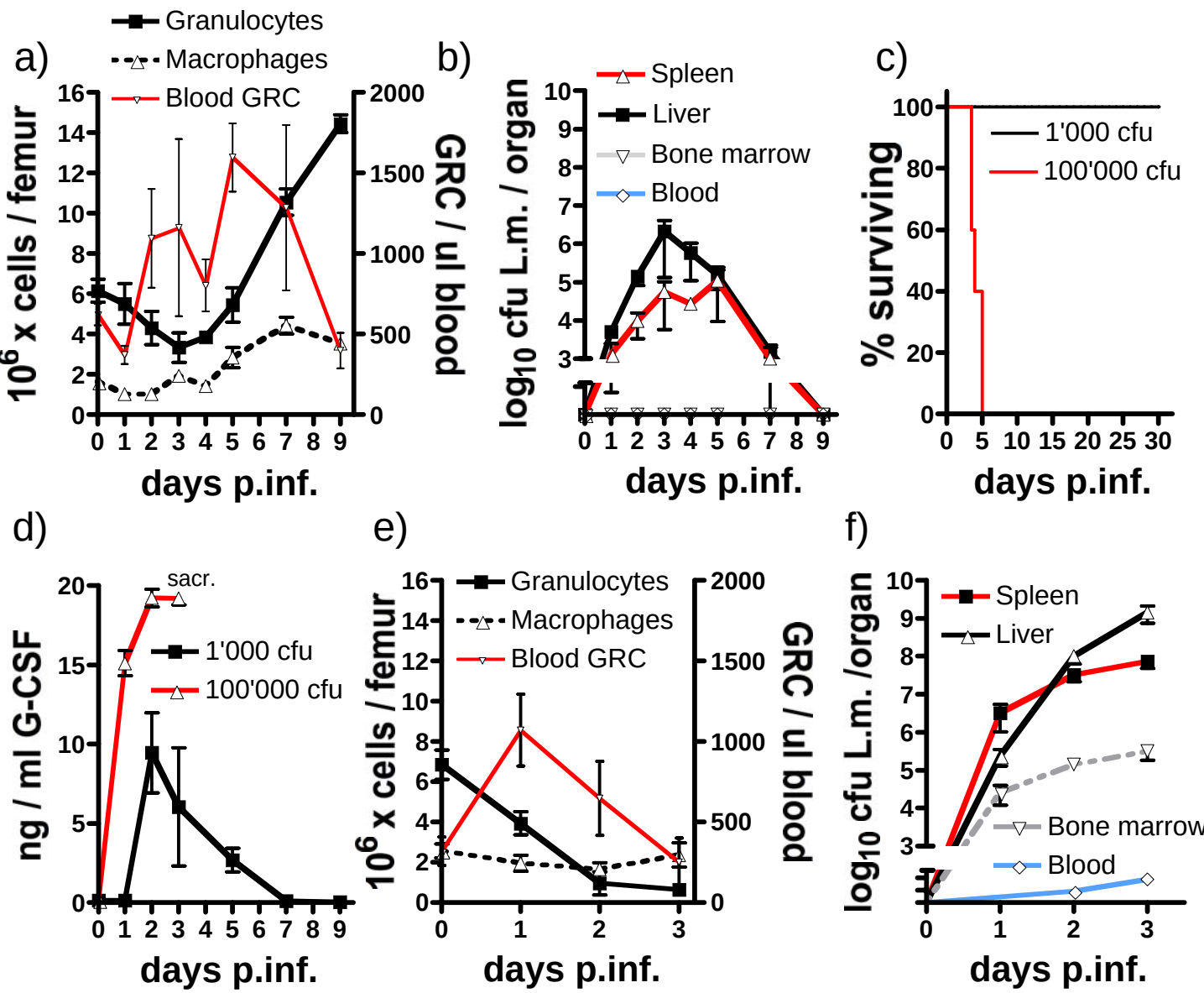

a)
Granulocytes
Macrophages
Blood GRC
10^6 x cells / femur
GRC / ul blood
days p.inf.
b)
Spleen
Liver
Bone marrow
Blood
$\log_{10}$ cfu L.m. / organ
days p.inf.
c)
1'000 cfu
100'000 cfu
% surviving
days p.inf.
d)
sacr.
1'000 cfu
100'000 cfu
ng / ml G-CSF
days p.inf.
e)
Granulocytes
Macrophages
Blood GRC
10^6 x cells / femur
GRC / ul blood
days p.inf.
f)
Spleen
Liver
Bone marrow
Blood
$\log_{10}$ cfu L.m. /organ
days p.inf.

Fig. 2a: Liver response during 10^3 c.f.u. L.m. i.v.

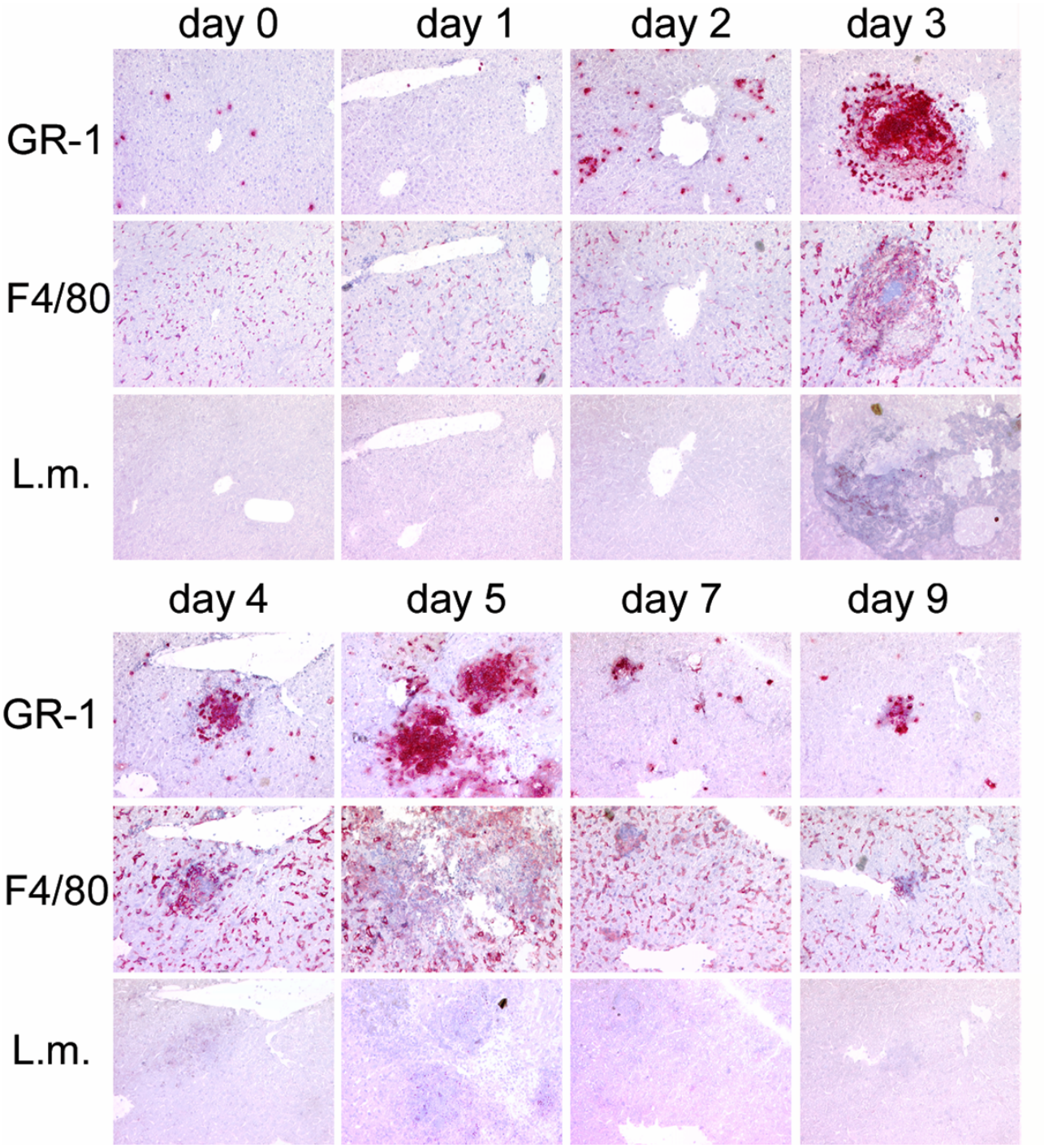


Fig. 2b: Splenic response during 10^3 c.f.u. L.m. i.v.

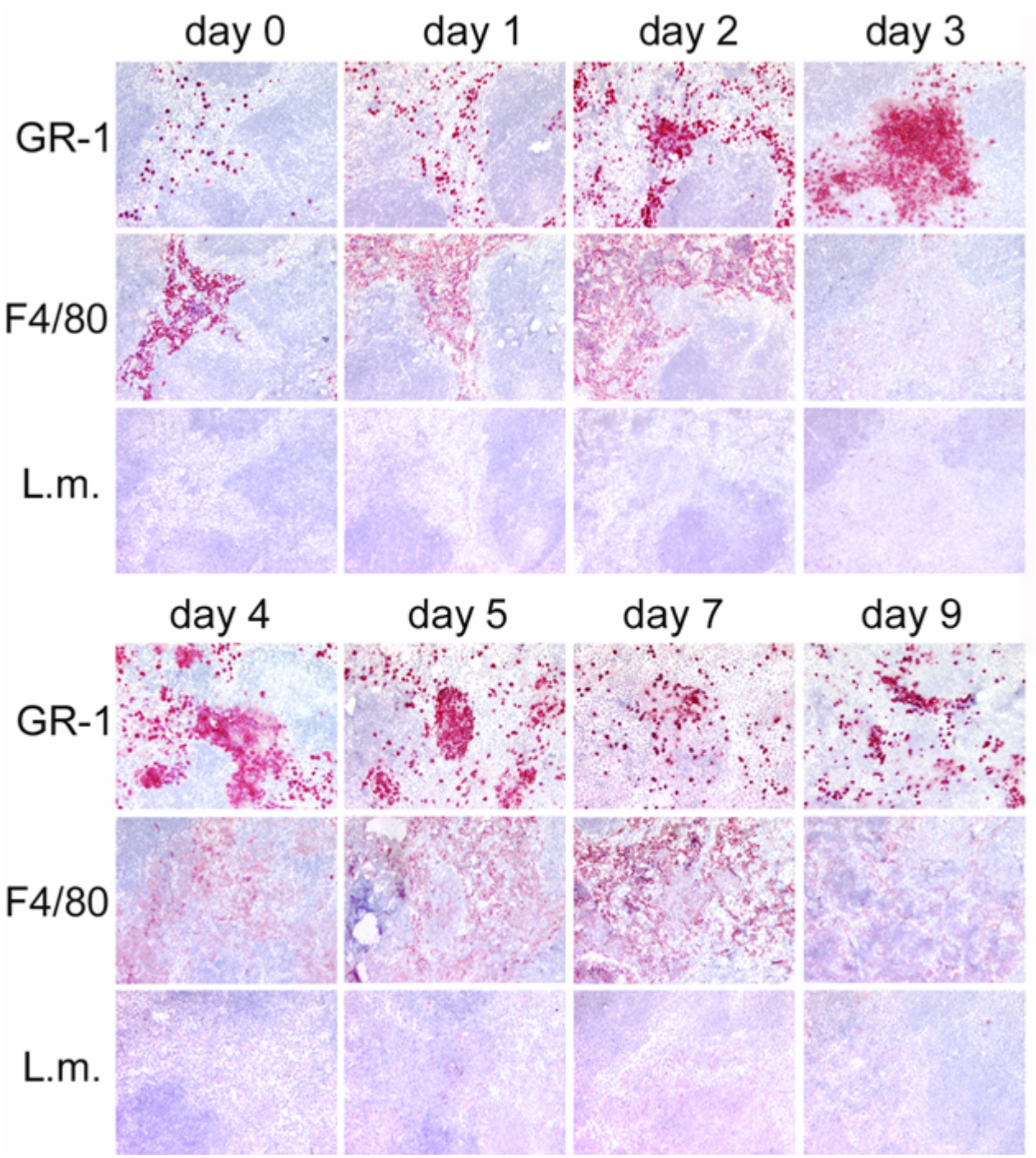
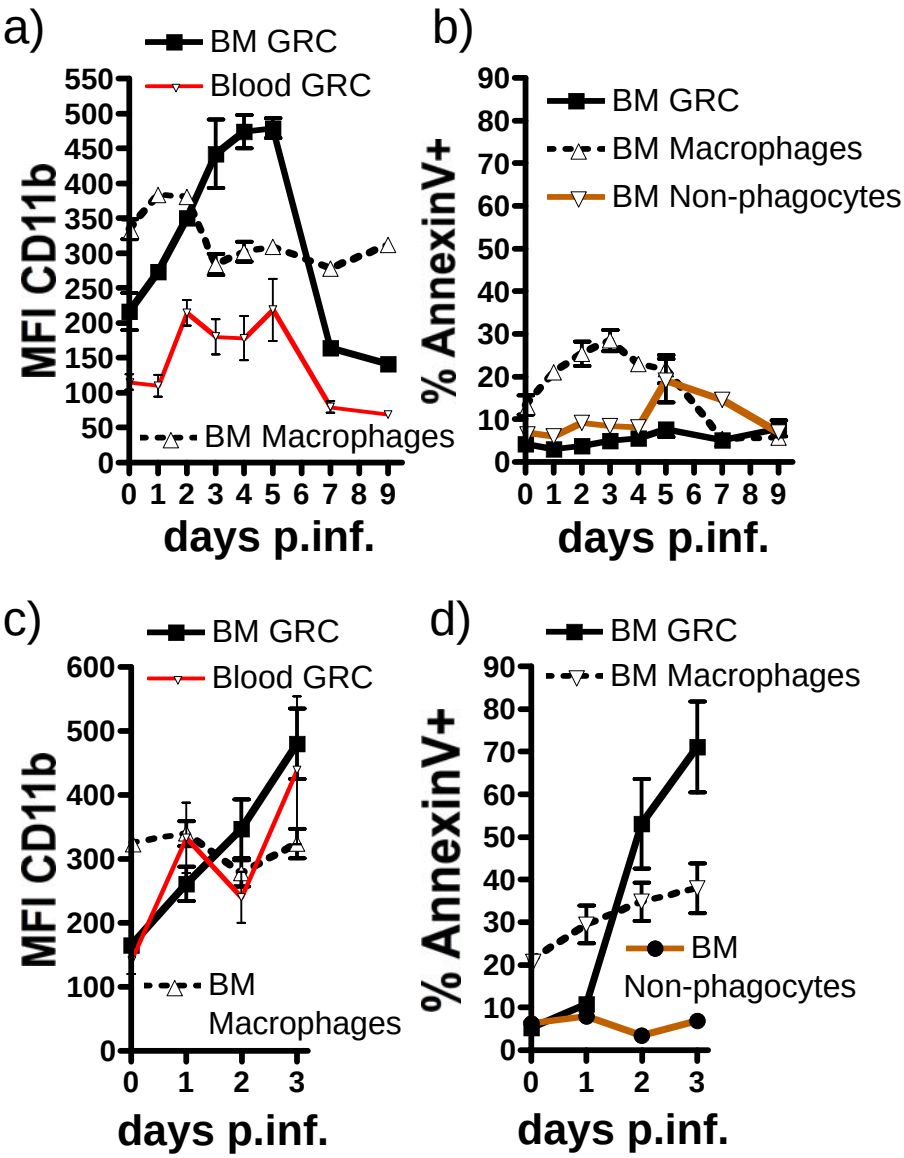


Fig. 3


a)
BM GRC
Blood GRC
BM Macrophages
MFI CD11b
550
500
450
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9
days p.inf.
b)
BM GRC
BM Macrophages
BM Non-phagocytes
% AnnexinV+
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
days p.inf.
c)
BM GRC
Blood GRC
BM Macrophages
MFI CD11b
600
500
400
300
200
100
0
0 1 2 3
days p.inf.
d)
BM GRC
BM Macrophages
BM Non-phagocytes
% AnnexinV+
90
80
70
60
50
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10
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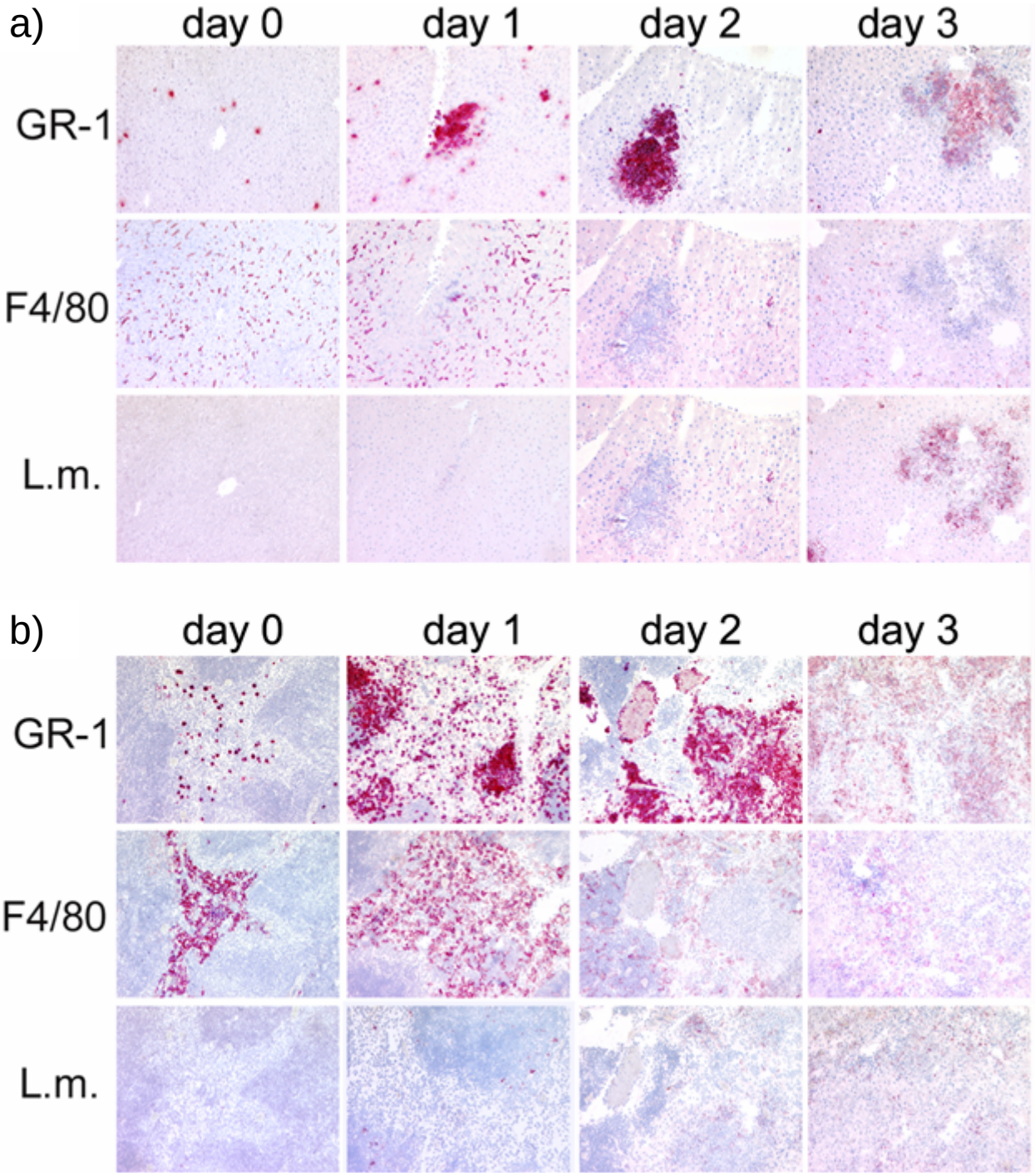
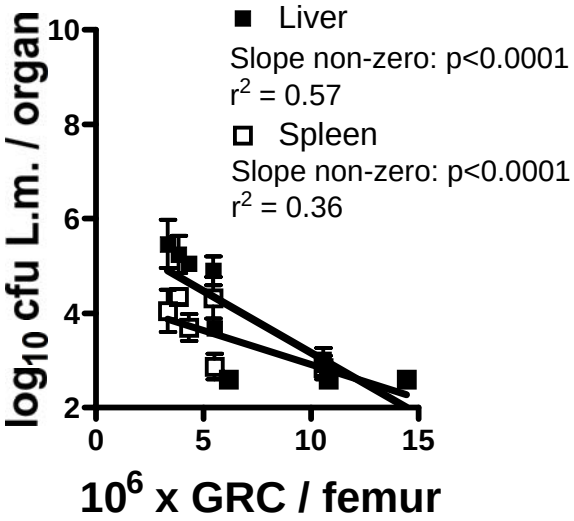
0 1 2 3
days p.inf.

Fig. 4: Hepatic and splenic response during 10^5 c.f.u. L.m. i.v.

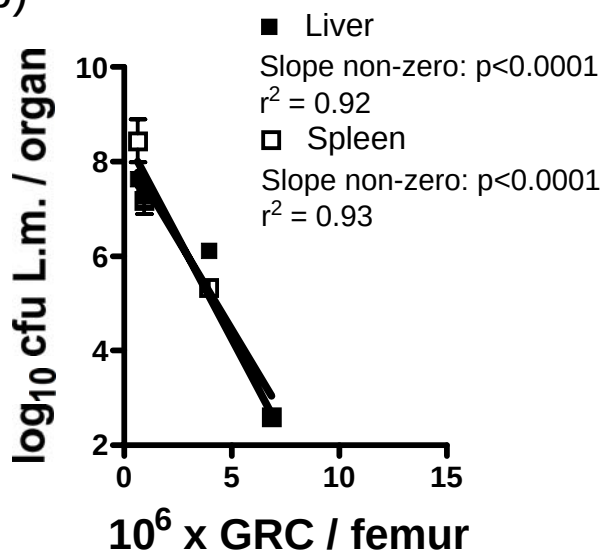


Supplementary Fig. 1

a)



b)



6. Increased susceptibility to bacterial super-infection as a consequence of innate antiviral responses

6.1 Abstract

The reason why severe localised or systemic virus infections enhance and aggravate bacterial super-infection is poorly understood. Here we show that virus-induced interferon type I caused apoptosis in bone marrow granulocytes, drastically reduced granulocyte infiltrates at the site of bacterial super-infection and caused up to 1000-fold higher bacterial titers in solid organs with decreased host survival. The finding that the innate anti-viral immune response reduces the anti-bacterial granulocyte defense offers an explanation of enhanced susceptibility to bacterial super-infection during viral disease.

6.2 Introduction

Better mechanistical insight into the process of facilitated bacterial super-infection during virus infection is needed since it is responsible for many of the 200'000 annual cases of life-endangering sepsis in the USA alone (Beadling and Slifka 2004). Although several local-acting mechanisms have been proposed (Bakaletz 1995; Black 2003; McCullers and Bartmess 2003; Okamoto, Kawabata et al. 2003), an explanation for generalized enhanced susceptibility to super-infection is still lacking. To address this, we have modelled a systemic bacterial super-infection during virus infection by combining well-studied model infections in mice. Lymphocytic choriomeningitis virus (LCMV) is a non-cytopathic RNA virus that rapidly spreads systemically and strongly induces type I interferon (IFN I) (Malmgaard, Salazar-Mather et al. 2002). Listeria monocytogenes (L.m.) and Staphylococcus aureus (S. aureus) are gram-positive bacteria that cause purulent infections. Both pathogens induce strong innate immune responses and attract granulocytes (Babior 2001) and subsequently T cell dependent macrophages whose role is to clear the bacteria by phagocytosis (Ebe, Hasegawa et al. 1999; Pamer 2004; Foster 2005). During agranulocytosis by cytotoxic drugs, irradiation or administration of monoclonal antibodies, hosts are rendered much more susceptible to bacterial infections (Rogers and Unanue 1993; Czuprynski, Brown et al. 1994; Conlan 1997). Here we show that during virus infection IFN I has an early severe granulotoxic effect that drastically increases susceptibility to bacterial super-infection.

6.3 Material and Methods:

Mice

Specific pathogen free (SPF) laboratory mice were from the institute of Labortierkunde of the veterinary facility of the University of Zurich. Experiments were performed according to Swiss veterinary law and institutional guidelines. C57BL/6 mice, IFN I receptor (IFNAR) knockout mice and relevant controls on 129/SvEv background were used.

Infections

Listeria monocytogenes (L.m.) strain 10403S grown overnight in Brain Heart infusion broth, washed two times in PBS and injected i.v. in 200 μl. Lymphocytic choriomengitis virus (LCMV) strain WE (from F. Lehmann-Grube, Heinrich Pette Institute, Germany) was grown on BHK cells and $2x10^6$ pfu were injected i.v. in 200 μl. Staphylococcus aureus Newman ATCC 25904 (S. aureus) was grown in Brain Heart Infusion medium, washed two times and frozen down. Thawed aliquots were washed and then used for infection was given i.v. in 200 μl at a dose of $2x10^7$ cfu per mouse. Salmonella typhimurium was grown in LB medium, and given i.v. in 200 μl at a dose of $1.2*10^4$ per mouse.

Bacterial titer

Bacterial titers were determined in homogenized halfs of spleen, the left lobe of the liver, the left lung or the whole left kidney in PBS with serial dilutions plated on brain heart infusion or blood agar plates.

Bone marrow aspirates and culture

Bone marrow cells were flushed with PBS from the femur and were stained for FACS analysis or cultured in RPMI with 10% FCS.

FACS and antibodies

Anti-Ly6G, GR1, CD11b, Annexin V and 7-Amino-Actinomycin-D (7-AAD) were obtained from BD, Basel. Cells expressing Ly6G / GR1 and CD11b are termed “GRC”. FSC/SSC gates were used to exclude debris in organ homogenates and peritoneal washings. Dihydrorhodamine 123 (DHR, Sigma) was used for measuring NADPH oxidase activity by measuring cellular fluorescence in FL1 channel (emission 534nm). A fixed number of fluroescent APC beads were used to quantify cell number per sample volume. Anti-GR1 (NimpR14) hybridoma was a generous gift from Dr Tacchini-Cottier, WHO, Geneva.

Histology

Histological samples were snap-frozen in Hanks medium and stained with an anti-L.m. rabbit serum (a gift from Prof J. Bille, Lausanne), Gr-1 (Pharmingen) or F4/80 (BM8, BMA) antibodies. Staining was developed using a goat anti-rat antibody (Caltag Laboratories) or goat anti-rabbit (Jackson Immuno Research) and an alkaline phosphatase-coupled donkey anti-goat antibody (Jackson Immuno Research) with naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid 2-methoxy anilide) phosphate and new fuchsin as a substrate. The presence of alkaline phosphatase activity yielded a red reaction product. The sections were counterstained with hemalum.

Determination of cytokine production

ELISA assays for murine IFN-α and IFN-β by PBL Biomedical Laboratories (Piscataway, NJ, USA) were used on sera and culture supernatants. G-CSF was measured with an ELISA from R&D (Minneapolis, USA).

Statistical analysis

In all figures, unpaired two-sided t test or one-way analysis of variance (ANOVA) was used. P values above 0.05 were not considered significant. * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$.

End notes:

A.A. Navarini and M. Recher have contributed equally to this work.

6.4 Results

When C57BL/6 mice were infected with LCMV-WE 2 days before super-infection with L.m. (Fig. 1a), they exhibited up to 1000-fold higher bacterial titers in liver and spleen at day 3 of the super-infection compared to control mice without LCMV infection (Fig. 1b) as well as drastically decreased survival (Fig. 1c). This early phase of L.m. infection is largely controlled by innate resistance through granulocytes (Rogers and Unanue 1993; Czuprynski, Brown et al. 1994; Conlan 1999; Seiler, Aichele et al. 2000; Wing and Gregory 2002), whereas after day 3, T cell-mediated control is crucial (Lane and Unanue 1972). Failure to control bacterial super-infection was not simply a consequence of virus replication, since bacterial elimination was even enhanced at day 5 after LCMV infection when virus titers were comparable or even higher than at day 3 (Supplementary Fig. 1a,b and data not shown). Together with existing evidence, this suggested that the underlying mechanism reflected a transient change of the early host anti-bacterial response. This finding was akin to the observation of an unexplained susceptibility to bacterial super-infection in the lung in the timeframe of day 2-5 after influenza A virus infection (Okamoto, Kawabata et al. 2003). In humans, bacterial super-infections become clinically apparent variably but usually starting as soon as 3-5 days after the onset of virus-induced clinical symptoms (Beadling and Slifka 2004). The recognized major role of granulocytes in controlling early L.m. infection (Rogers and Unanue 1993; Czuprynski, Brown et al. 1994; Conlan 1997; Pamer 2004) was confirmed by depletion of granulocytes at day 1 after bacterial infection by administration of an anti-GR1 antibody (Fig. 1b). Thus, increased susceptibility due to viral pre-infection could be mimicked by physical absence of granulocytes. To determine whether granulocytes were indeed involved and affected, bone marrow granulocytes were quantified by FACS three days after super-infection. Granulocytes were strongly reduced in LCMV pre-infected mice compared to control mice up to day 5 (Fig. 1d).

Next, granulocyte kinetics after LCMV infection in absence of bacterial super-infection were measured. Granulocyte numbers in bone marrow of C57BL/6 mice were analysed at different time-points after LCMV-WE infection (Fig. 1e). After an initial small increase, granulocyte numbers dropped to about 30% of the original number from 2-5 days after LCMV infection. This correlated with induction of granulocyte apoptosis (Fig. 1e). Thereafter, granulocyte numbers normalized until day 9. In a study analyzing influenza infection in monkeys as well as in another with

human patients, a reduction of blood polymorphonuclear leukocytes has been reported at day 3 after admission to the hospital (Berendt and Hall 1977; Lewis, Gilbert et al. 1986). The fate of other cell types during LCMV infection was also investigated by immunohistology. In spleen, macrophages increased somewhat at day 5 after LCMV infection, while liver macrophages remained unchanged during the entire period analysed (Supplementary Fig. 2a). NK cell numbers in the bone marrow stayed at low levels until day 5, then strongly increased in numbers (Supplementary Fig. 2b). T cell numbers were low until day 7, when the expected LCMV-associated T cell proliferation could be demonstrated (Supplementary Fig. 2b). $B220^+$ B cells decreased (Borrow, Hou et al. 2005) comparably to granulocytes early during LCMV infection but did not recover to original numbers during the 15 days studied, reflecting the known immunopathological destruction of lymphoid tissue (Moskophidis, Pircher et al. 1992) (Supplementary Fig. 2b). Thus, only granulocyte kinetics correlated with the relatively short time period of virus-induced failure to control bacterial super-infection.

To determine whether virus-induced granulocytopenia might be associated with excessive activation, expression of the β_2-integrin CD11b on the surface of bone marrow granulocytes after virus infection was measured (Fig. 2a). Granulocytes were activated by 24 hours after LCMV infection. At day 7 after virus infection, CD11b surface expression normalized. Both phagocytosis and superoxide production by bone marrow granulocytes was increased in LCMV-infected compared to untreated animals (Fig. 2b,c). To determine whether the observed state of pre-activation resulted in heightened cell damage upon contact with live bacteria, we measured the percentage of 7-Amino-Actinomycin-D^+ granulocytes after 1 hour of co-incubation with L.m. Dead granulocytes were increased 2.5 fold after virus infection and bacterial challenge vs. untreated, while both virus and bacterial infection alone also induced granulocyte damage (1.7 and 1.9 fold of untreated) (Fig. 2d and Fig. 1e).

Our results so far demonstrated that granulocyte activation and induction of apoptosis correlated with a failure to effectively counter bacterial super-infection. Mechanistically, the lowered granulocyte reservoir in the bone marrow could limit the effective granulocyte homing to the site of bacterial super-infection. C57BL/6 mice were therefore challenged with a high dose of L.m. at day 3 of LCMV infection. Numbers of infiltrating granulocytes were analysed in spleen and liver four hours after L.m. injection. Virus-infected animals showed three-fold less granulocytes in

both organs (Fig. 2e). In an additional experiment, thioglycolate, known to provoke rapid recruitment of granulocytes into the peritoneum (Baron and Proctor 1982), was injected i.p. at day 3 of LCMV infection. Four hours later, LCMV-infected mice demonstrated 50% less granulocytes in the peritoneal cavity than control animals (Fig. 2f).

The observed bone marrow granulocytopenia could reflect several mechanisms, reduction by apoptosis in the bone marrow and/or emigration and accumulation in the periphery. The third possibility, i.e. reduced induction of granulopoiesis, was an unlikely explanation as strongly elevated expression of granulocyte colony stimulating factor (G-CSF) at the time of granulocyte depletion was measured (Fig. 1e). Assessment of apoptosis on bone marrow cell suspensions after LCMV infection revealed increased granulocyte apoptosis starting from day 2 after infection. This inversely correlated with the number of granulocytes present in the bone marrow (Fig. 1e). Induction of apoptosis was specific for granulocytes, as non-granulocytic bone marrow cells did not show elevated apoptosis at day 3, the time-point of maximal granulocyte apoptosis (Supplementary Fig. 2c). We then tested whether granulocytes showed enhanced emigration and accumulation in the periphery by quantifying granulocytes in blood, spleen and liver by FACS and immunohistology (Supplementary Fig. 2e). Blood granulocyte numbers dropped early during virus infection, then stayed low and increased only after day 5 (Supplementary Fig. 2d). In spleen, detectable granulocytes approximately doubled by day 2 after LCMV infection, corresponding to about two million granulocytes per spleen. As we observed a loss of approximately 70% of the total 65 million granulocytes (Babior 2001) in the entire bone marrow, enhanced migration could not explain bone marrow granulocyte loss. Granulocyte numbers remained unchanged in the liver (Supplementary Fig. 2e). Taken together, activation-associated granulocyte death following virus infection was the main mechanism for the observed bone marrow granulocytopenia.

Next, we tested whether granulocyte apoptosis was a consequence of antiviral mediators produced early during LCMV infection. Type I interferon (IFN I) production after LCMV infection peaked at day 1 after infection and inversely predicted bone marrow granulocyte numbers (Fig. 3a). To demonstrate causal relationship, IFN I receptor knockout mice ($IFNAR^{-/-}$) were infected with LCMV and bone marrow granulocyte numbers were measured at day 3 when T cell responses had not been

mounted yet and viral titers were equal in both strains (Binder, Fehr et al. 1997). IFNAR$^{-/-}$ did not exhibit a reduction of granulocytes in the bone marrow whereas wild-type mice had 12-fold reduced granulocyte numbers compared to untreated animals (Fig. 3b). Furthermore, IFNAR$^{-/-}$ showed no increase in granulocyte apoptosis following LCMV infection *in vivo* (Fig. 3c). Correspondingly, bone marrow cell suspensions of naïve wild-type but not IFNAR$^{-/-}$ treated *in vitro* with recombinant type I interferon for 72 hours revealed a dose-dependent induction of granulocyte apoptosis (Fig. 3d).

To confirm the crucial role of virus-induced IFN I in impairing granulocyte-mediated control of bacterial infection, IFNAR$^{-/-}$ and wild-type mice were LCMV-infected and two days later super-infected with L.m. Another two days later, wildtype mice displayed 1000-fold higher bacterial titers than IFNAR$^{-/-}$ in spleen, and about 20-fold higher titers in liver (Fig. 4a). This correlated with drastically lower granulocyte infiltration in the periphery in LCMV infected wildtype mice (Fig. 4c,d and lower magnifications in supplementary Fig. 3). The virus-induced bone marrow granulocytopenia was not complete but became functionally limiting during bacterial super-infection, where large numbers of bone marrow-derived granulocytes are required to control bacterial lesions. During these experiments, granulocyte numbers were also monitored in blood. While naïve mice and LCMV pre-infected IFNAR$^{-/-}$ animals increased granulocytes in blood during L.m. infection, LCMV-infected wildtype animals were unable to mount reactive granulocytosis upon super-infection (Fig. 4b). Recent reports have implicated a detrimental effect of IFN I during L.m. infection partly by acting on T cells (Auerbuch, Brockstedt et al. 2004; Carrero, Calderon et al. 2004; O'Connell, Saha et al. 2004; Carrero, Calderon et al. 2006). Although at the dose of L.m. and in the 129/SvEv background we were using, these L.m. titer differences were marginal, our findings pointed in the same direction as the results of these recent studies. The toll-like receptor 3 ligand Poly(I:C) has been described to increase susceptibility to L.m. infection (O'Connell, Saha et al. 2004). We therefore analysed whether Poly(I:C) affects the granulocyte compartment similar to a LCMV pre-infection. IFNAR-competent and -deficient mice were infected with $5x10^3$ cfu L.m. and treated with 200μg Poly(I:C) at the day of infection. As described, this treatment resulted in increased L.m. titers only in IFNAR-expressing animals at day 3 of the L.m. infection (Supplementary Fig. 4a). When bone marrow granulocytes were analysed, Poly(I:C) treated wild-type animals showed increased activation

(Supplementary Fig. 4b) and apoptosis (Supplementary Fig. 4c) within the granulocyte compartment that was reduced in numbers (Supplementary Fig. 4d,e,f). Poly(I:C) treatment alone also resulted in IFNAR-dependent reduction of granulocyte numbers and increased apoptosis in the bone marrow (Supplementary Fig. 4g,h).

To determine whether susceptibility to super-infection extended to other bacteria, the mostly extracellular bacterium S. aureus was analysed. IFNAR$^{-/-}$ mice were pre-infected with LCMV and challenged two days later with S. aureus. After 24 hours, LCMV-infected wildtype mice demonstrated drastic clinical disease as well as 100-fold higher bacterial titers in lung and kidney when compared to IFNAR$^{-/-}$ mice (Fig. 4e). When LCMV-infected C57BL/6 mice were tested to exclude mouse-strain dependence of the observation, also 100-fold increased bacterial organ titers were measured in LCMV pre-infected animals compared to control mice (not shown). To determine whether virus-induced granulocytopenia would also heighten susceptibility to a gram-negative super-infection, we tested Salmonella typhimurium (S. typhim.) in C57BL/6 mice (Conlan 1997). At day 2 of LCMV infection, $1.2*10^4$ cfu S. typhim. were given intravenously. Bacterial titers were elevated at day 3 in spleen and liver in LCMV pre-infected compared to unpretreated mice (Fig. 4f).

To test whether bacterial super-infection at the onset of bone marrow granulocyte recovery after LCMV infection would be cleared normally, we infected C57BL/6 mice with 5000 L.m. at day 5 after LCMV. Interestingly, and perhaps, as might be expected at this later timepoint, mice cleared these infections better than controls (Supp. Fig. 1).

6.5 Discussion

The virus-induced suppression of anti-bacterial immunity is characterized by type I interferon production followed by apoptosis of bone marrow granulocytes, which in turn causes impaired granulocyte emigration to sites of bacterial infection. This granulocytopenia is not complete but became functionally limiting during super-infection, where large numbers of bone marrow-derived granulocytes were rapidly required to control L.m., S. aureus or S. typhimurium. Since LCMV is not cytopathic in its physiological murine host, direct cell destruction by replicating virus is not necessary for enhancement of bacterial super-infection.

In addition to systemic effects, local virus-induced alterations including enzymatic uncovering of bacterial binding sites on host cells (McCullers and Bartmess 2003), lysis of epithelial cells by cytopathic viruses (Black 2003), that may break antibacterial barriers (Bakaletz 1995), and enhanced bacterial binding to virus-encoded receptors that are expressed on infected host cells (Okamoto, Kawabata et al. 2003) may additionally facilitate bacterial super-infection. Interferon-induced granulocyte apoptosis could also explain often observed sepsis following burn injuries or ischemic stroke (Davenport, Dennis et al. 1996; Hotchkiss, Coopersmith et al. 2005; Sharma, Harish et al. 2006). From the virus' point of view, the induction of innate antiviral IFN I, by depleting granulocytes, may also diminish later adaptive immune responses (Slifka, Homann et al. 2003; Hahm, Trifilo et al. 2005; Molesworth-Kenyon, Oakes et al. 2005) and may thus favour virus persistence. Neutropenia is a regular symptom of virus infections such as EBV (Hammond, Harlan et al. 1979) and HIV (Colson, Foucault et al. 2005). Interferon type I as a therapeutic agent has also been shown to induce granulocytopenia (Urbaniak, Halliday et al. 1978; Gutterman, Fine et al. 1982), and Hepatitis C and B patients receiving therapeutic type I IFN have been shown to have an increased risk for bacterial super-infections when rendered neutropenic by the treatment (Hoofnagle and di Bisceglie 1997; Puoti, Babudieri et al. 2004).

Taken together, our findings suggest that virus-induced granulocytopenia may critically contribute to bacterial super-infections. Efforts to block this effect, possibly with preventive G-CSF application or perhaps even cautious anti-IFN I treatments during the acute phase of the virus infection could ameliorate the outcome of the described clinical constellation.

6.6 Author contributions

A. Navarini performed most experiments and wrote the manuscript. M. Recher performed the smaller part of the experiments, provided experimental help and helped with writing the manuscript. Assistance with experiments as well as intellectual help was provided by K. Lang, P. Georgiev, S. Meury, A. Bergthaler and L. Flatz. Histology was performed by Prof. B. Odermatt. R. Landmann and J. Bille assisted with crucial reagents. The work was performed in the laboratory of Prof. R.M. Zinkernagel and Prof. H. Hengartner who guided the work and manuscript.

6.7 Figure legends:

Fig. 1: Increased susceptibility to bacterial infection early during LCMV-induced bone marrow granulocytopenia

(A) Experimental protocol. (B) Bacterial titers in spleen and liver at day 3 of infection. C57BL/6 mice were infected with $2x10^6$ pfu LCMV-WE i.v. 48 hours before infection with $5x10^3$ cfu (1/5 LD_{50}) Listeria monocytogenes (L.m.) i.v. For comparison, 120μg of granulocyte-depleting antibody anti-GR1 NimpR14 was given i.v. at day 1 after L.m. infection in an additional group of mice (mean +/- SEM of 4-6 animals per group, one of three experiments). (C) C57BL/6 mice were infected with LCMV at day -2, and infected with $1x10^3$ (1/10 LD_{50}) or $5x10^3$ (1/2 LD_{50}) cfu L.m. at day 0. Survival was monitored twice daily (n=9-10 per group). (D) Bone marrow granulocytes (GRC, $Ly6G^+CD11b^+$) cell numbers measured by FACS at day 5 after LCMV and day 3 after L.m. super-infection (n=4-6 animals per group, one of three experiments). (E) Bone marrow GRC (black) of C57BL/6 mice after infection with $2x10^6$ pfu LCMV-WE i.v., percentage of Annexin V^+ of GRC (red) (mean +/- SEM, n=3-4 per group, one of two experiments with similar results). G-CSF concentration in serum (blue) (mean +/- SEM, n=3-4 per group).

Fig. 2: Activation-associated cell death of bone marrow GRC during LCMV infection leads to impaired granulocytic infiltration

(A) Upregulation of CD11b surface expression on bone marrow GRC during LCMV infection (n=3-4 animals per timepoint, mean fluorescence intensity +/- SEM). (B) Phagocytosis of CFSE-labelled L.m. *in vitro* by BM GRC (MOI 20:1) at day 2 after LCMV infection after 1 hour of co-incubation. Mean fluorescence intensity of 7-AAD-neg. GRC (one of two experiments with similar results). (C) NADPH Oxidase activity in 7-AAD-neg. BM GRC as measured by DHR123 fluorescence (534nm) *in vitro* upon co-incubation with L.m. for 1 hour at day 2 after LCMV infection (one of three experiments with similar results). (D) Percentage of dead ($7\text{-}AAD^+$) bone marrow GRC from LCMV pre-infected C57BL/6 mice or control mice after incubation with or without L.m. *in vitro* for 1 hour. (one of two experiments with similar results). (E) Numbers of hepatic and splenic GRC 4 hours after L.m. challenge (10^7 cfu i.v.) in C57BL/6 mice at day 3 after $2x10^6$ pfu LCMV infection (one of two experiments with similar results). (F) Numbers of peritoneal GRC 4 hours after administration of 1ml thioglycolate i.p. in

C57BL/6 mice at day 3 after LCMV infection (one of two experiments with similar results).

Fig. 3: Type I interferon dependent bone marrow GRC apoptosis
(A) Serum interferon type I in LCMV infected C57BL/6 mice (n=3-4, mean +/- SEM). (B) Numbers of bone marrow GRC of IFNAR$^{-/-}$ and control mice at day 3 after LCMV infection or without virus infection (one of two experiments with similar results). (C) Percentages of apoptotic (Annexin V^{+}) bone marrow GRC of IFNAR$^{-/-}$ and control mice at day 3 after LCMV infection or without virus infection (n=3 animals per group, mean +/- SEM, one of two experiments with similar results). (D) Apoptotic GRC (Ly6G^{+}) after 72 hours *in vitro* bone marrow culture of wildtype and IFNAR$^{-/-}$ mice with increasing amounts of recombinant interferon α or β (one of two similar experiments).

Fig. 4: Interferon type I dependent granulocytopenia and absence of granulocyte infiltrates at the site of bacterial infection
(A) IFNAR$^{-/-}$ mice and wild-type mice were infected with LCMV 48 hours before super-infection with 5x10^{3} L.m. Two days later, L.m. titers were determined (n=3-4 per group, one of three comparable experiments is shown, mean +/- SEM). (B) Numbers of peripheral blood GRC (n=3-4 per group, one of two experiments, mean +/- SEM). (C) Immunohistochemistry of GRC (GR-1) in liver and (D) spleen and of L.m. (rabbit anti-L.m.) in sequential sections at day 2 after super-infection with L.m. in LCMV infected IFNAR$^{-/-}$ or control wildtype mice (two experiments with similar results). (E) IFNAR$^{-/-}$ mice and control mice were infected with LCMV at day -2 and super-infected at day 0 with 2x10^{7} cfu S. aureus. 24 hours later, S. aureus titers were determined (n=3-4 per group, mean +/- SEM). (F) C57BL/6 mice infected with LCMV at day -2 were super-infected with 1.2*10^{4} S. typhimurium i.v. and bacterial titers determined at day 3.

Supplementary Fig. 1: When super-infection occurs at the onset of bone marrow granulocyte recovery, L.m. clearance is enhanced

(A) Experimental protocol (B) C57BL/6 mice were infected with LCMV at day -5, and infected with $5x10^3$ cfu L.m. at day 0. At day 3, bacterial titers were determined in spleen and liver (n= 3-4 per group, one experiment).

Supplementary Fig. 2: LCMV-induced apoptosis is specific for bone marrow GRC

(A) Time-course of splenic and hepatic macrophages stained with F4/80 after infection of C57BL/6 mice with $2x10^6$ pfu LCMV WE (representative examples, one experiment, n=3). (B) Numbers of bone marrow T ($CD3e^+$), B ($B220^+$) and NK cells ($NK1.1^+$) after infection with $2x10^6$ pfu LCMV WE (one experiment, mean +/- SEM). (C) Percentage of apoptotic (Annexin V^+) bone marrow GRC and non-GRC at day 3 of LCMV infection or in naïve mice (one of three experiments with similar results, mean +/- SEM). (D) Blood GRC numbers during $2x10^6$ pfu LCMV WE infection of C57BL/6 mice. (one of two experiments with similar results, mean +/- SEM) (E) Immunohistochemistry of GRC (GR-1) during LCMV infection of C57BL/6 mice (representative examples, one experiment, n=3).

Supplementary Fig. 3: Interferon type I dependent granulocytopenia during LCMV infection leads to absence of granulocytes, but not macrophages, at the site of infection

At day 2 after L.m. super-infection of LCMV-WE pre-infected mice, livers of wildtype and $IFNAR^{-/-}$ mice were stained for the presence of granulocytes (GR-1), tissue macrophages (F4/80) and L.m. antigen (representative examples, n=3-4, two experiments with similar results).

Supplementary Fig. 4: Poly(I:C) treatment upon L.m. infection leads to type I IFN dependent heightened BM GRC activation, apoptosis, decreased BM GRC numbers and elevated L.m. organ titers. Poly(I:C) treatment in naïve mice reduces granulocyte numbers in bone marrow and induces apoptosis.

(A) L.m. liver titers at day 3 after infection of $IFNAR^{-/-}$ or wild type mice with $5x10^3$ L.m. with or without concomitant administration of 200μg Poly(I:C) i.v. at the day of infection (n=3 mice per group, mean +/- SEM, one of two experiments with similar results). (B) CD11b surface expression on bone marrow GRC

(Ly6G^{+}CD11b^{+}) (n=3 per group, mean +/- SEM, one of two experiments with similar results). (C) Percentage of apoptotic (Annexin V^{+}) bone marrow GRC and (D) percent and (E) numbers of bone marrow GRC at day 3 after L.m. infection (n=3 per group, mean +/- SEM, one of two experiments with similar results). (F) Representative FACS plots of (D). (G) Numbers of bone marrow GRC and (H) percentage of apoptotic GRC are shown 48 hours after administration of 200 μg Poly(I:C) i.v. (n=3 per group, one experiment).

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Fig. 1: Increased susceptibility to bacteria infection early during LCMV-induced bone marrow granulocytopenia

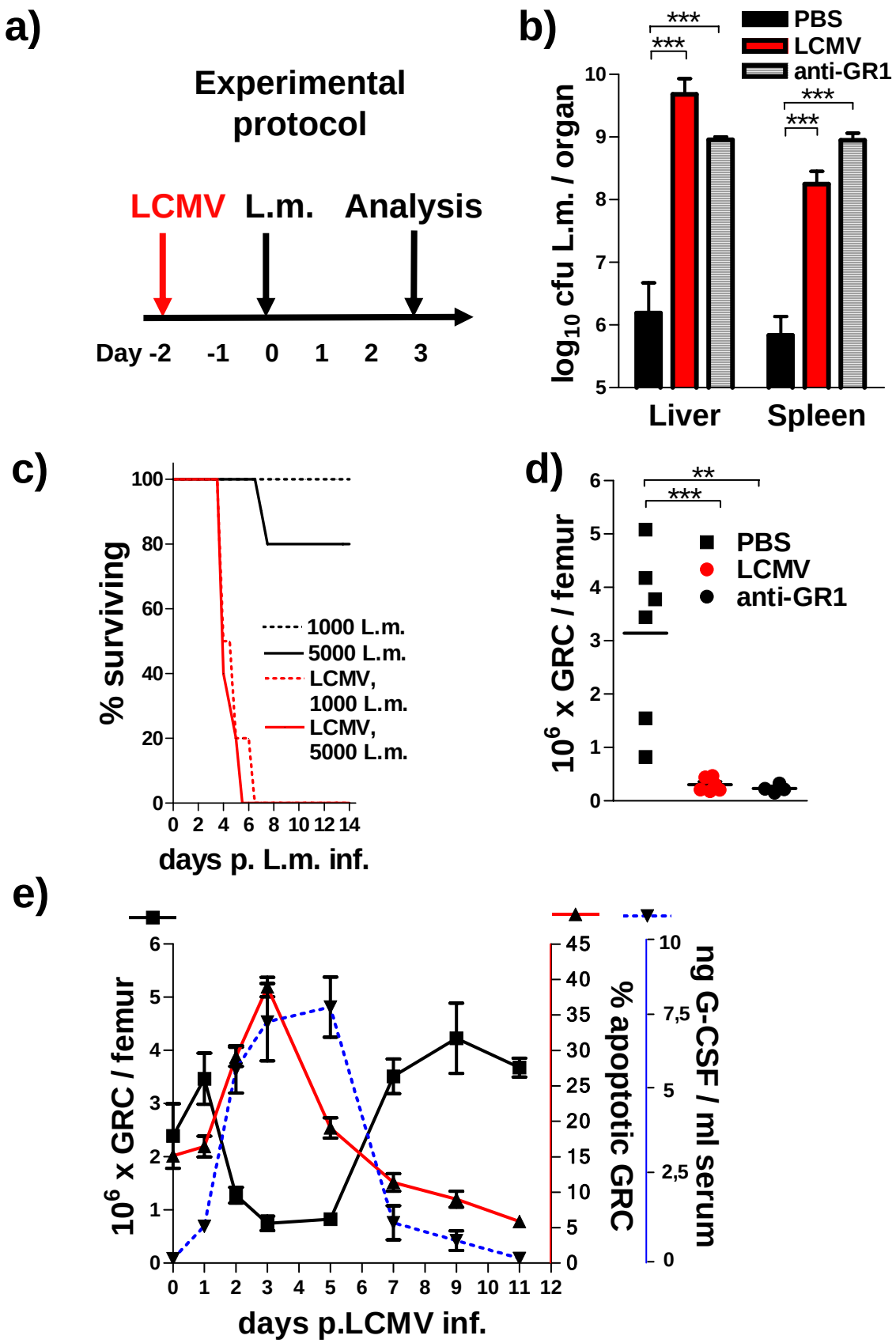


Fig. 2: Activation-associated cell death of bone marrow GRC during LCMV infection leads to impaired granulocytic infiltration

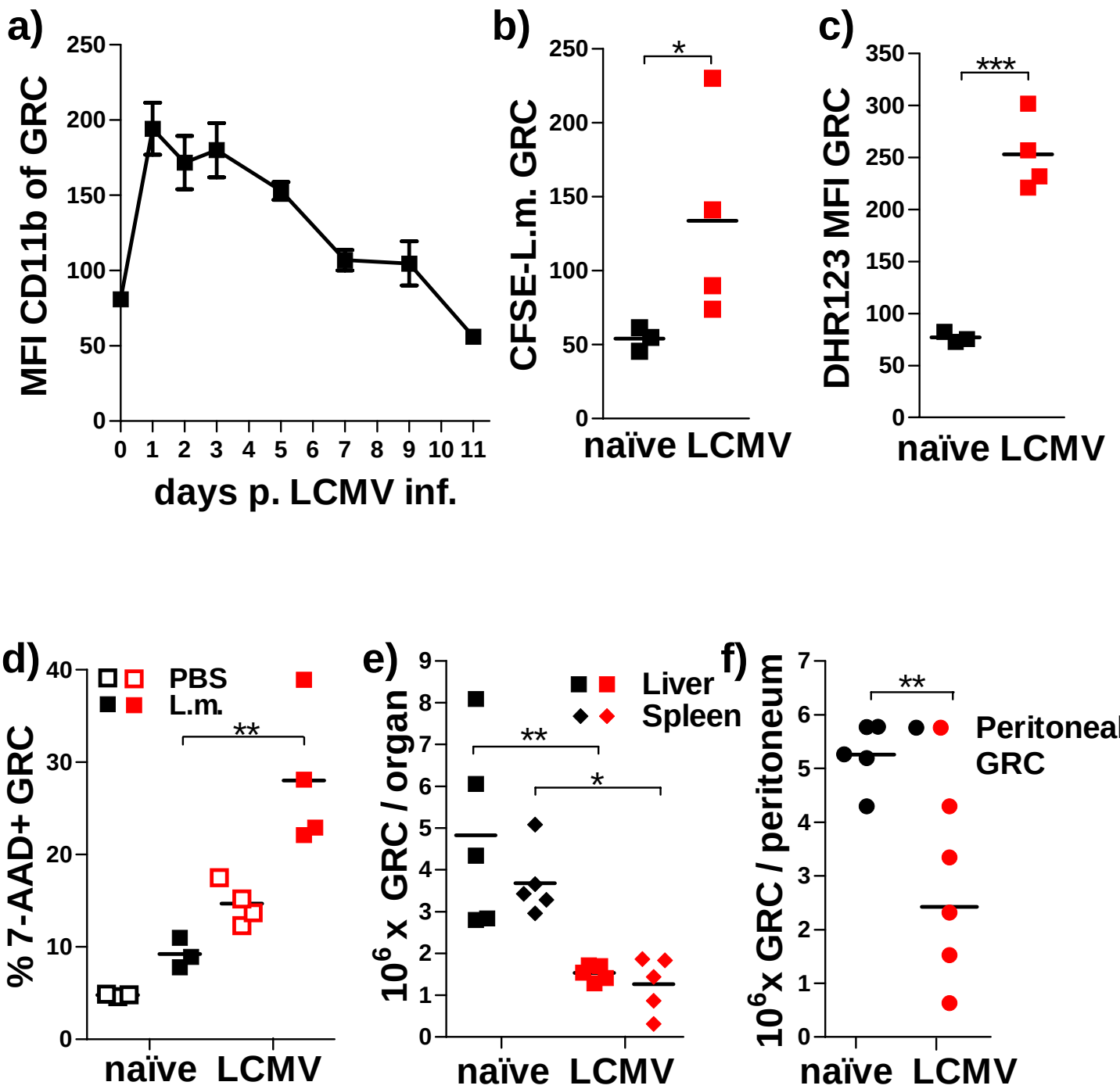


Fig. 3: Type I interferon dependent bone marrow GRC apoptosis

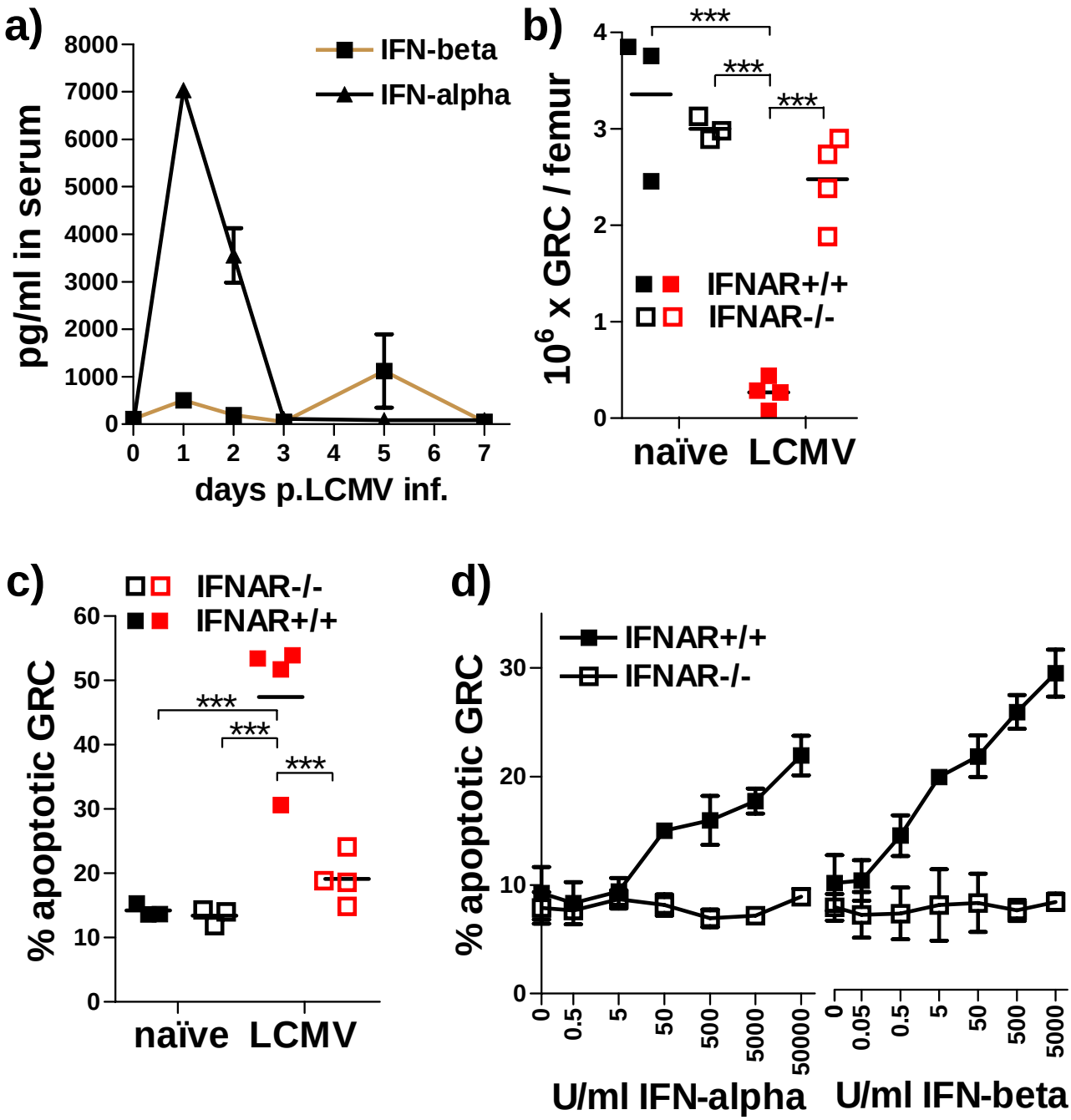
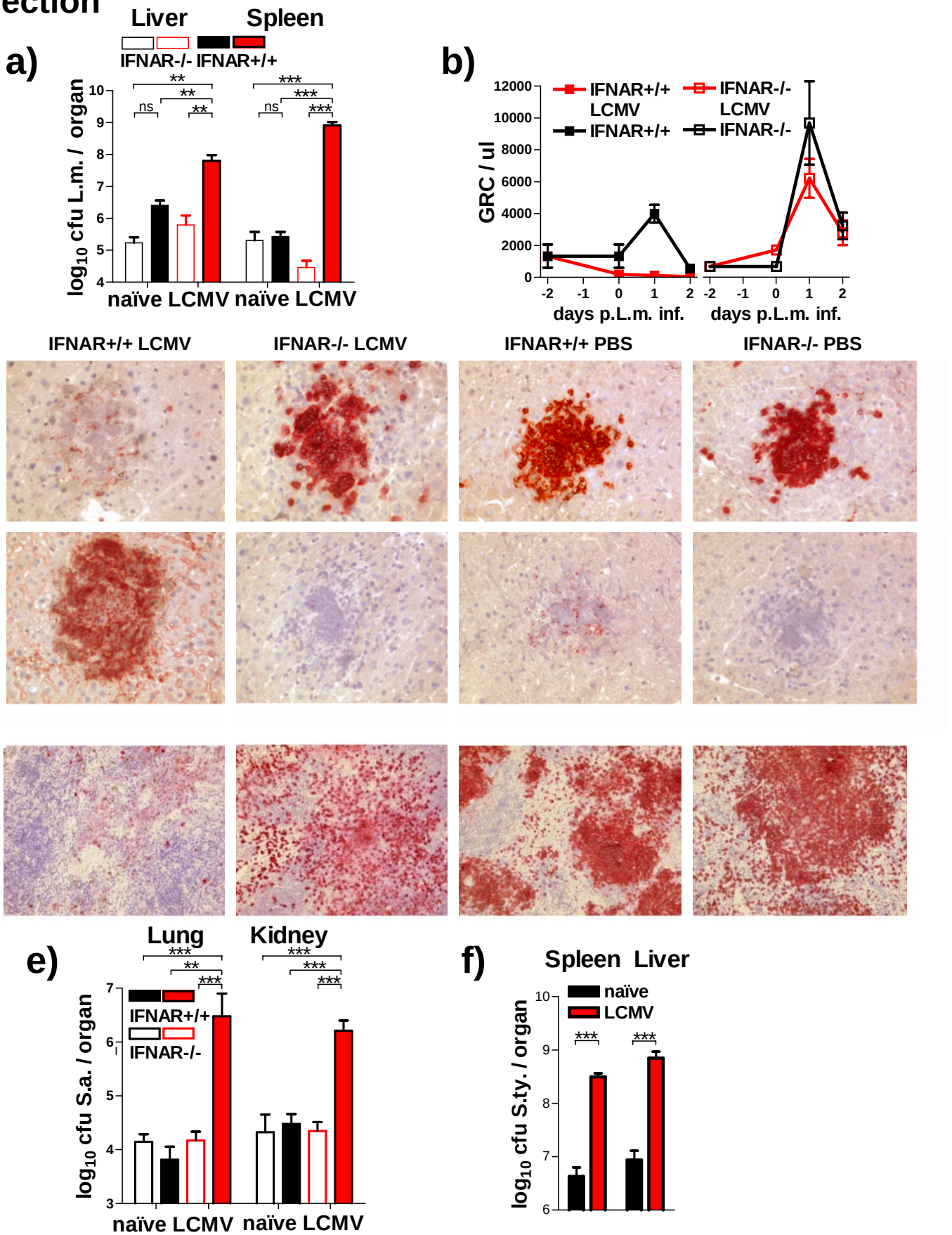
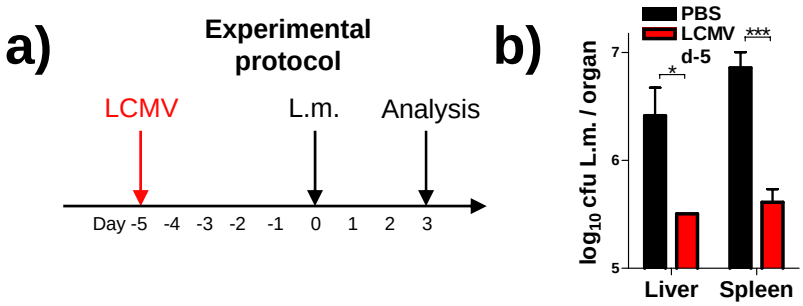


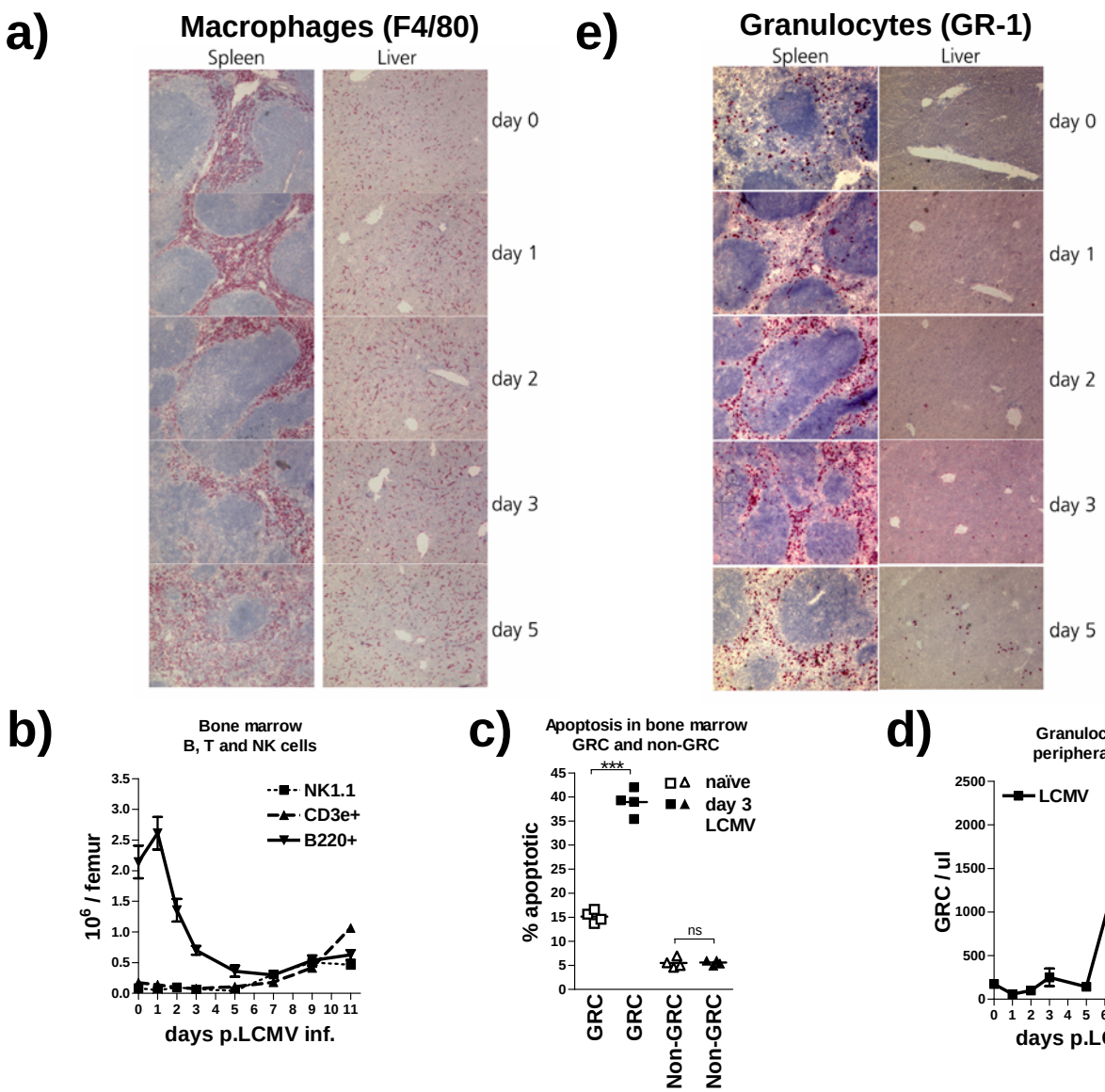
Fig. 4: Interferon type I dependent granulocytopenia and absence of granulocyte infiltrates at the site of bacterial infection



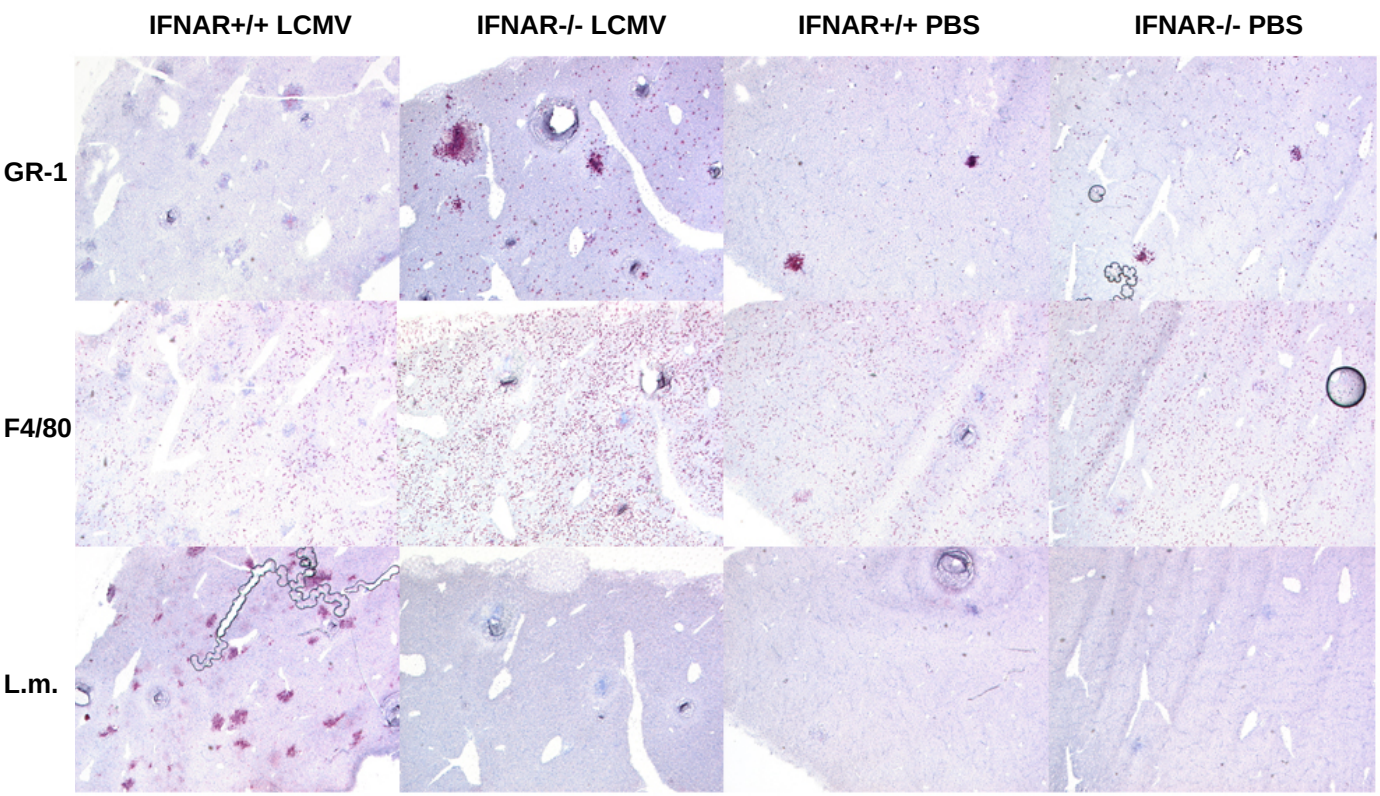
Supplementary Fig. 1: When super-infection occurs at the onset of bone marrow granulocyte recovery, L.m. clearance is enhanced



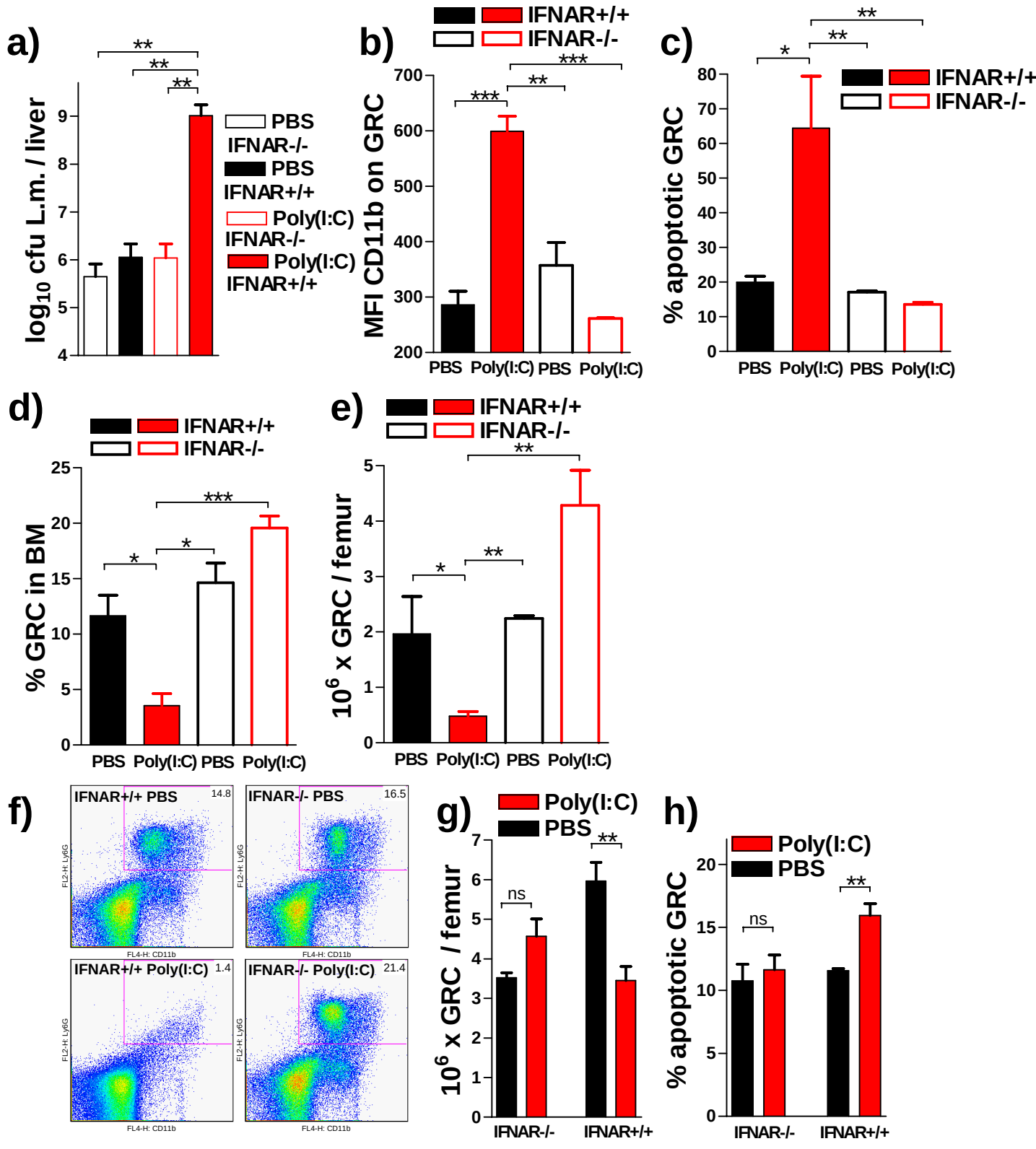
Supplementary Fig. 2: LCMV-induced apoptosis is specific for bone marrow GRC



Supplementary Fig. 3: Interferon type I dependent granulocytopenia during LCMV infection leads to absence of granulocytes, but not macrophages, at the site of infection



Supplementary Fig. 4: Poly(I:C) treatment upon L.m. infection leads to type I IFN dependent heightened BM GRC activation, apoptosis, decreased BM GRC numbers and elevated L.m. liver titer. Poly(I:C) treatment in naïve mice reduces granulocyte numbers in bone marrow and induces apoptosis.



7. Disruption of the bone marrow granulocyte supply and uncontrolled bacterial proliferation by TLR2 activation

7.1 Abstract

Bacterial infections are normally quickly controlled by granulocytes. After phagocytosis and exertion of their antibacterial function, granulocytes undergo cell death, forming pus. Therefore, rapid and constant supply from the bone marrow is needed to control bacterial abscesses. Here we show that when TLR2/6 ligands reach the circulation and spread systemically during an innocuous bacterial infection, the essential granulocyte supply in the bone marrow can be activated and destroyed, resulting in unchecked bacterial proliferation, sepsis and death. This mechanism could be relevant in the pathophysiology of sepsis.

7.2 Introduction

The first infiltrating cells to counter a bacterial infection are the granulocytes. They emigrate from the bone marrow to the blood stream and enter target tissues. In circulation, granulocytes have a short half-life of about 8 hours. The bone marrow contains a great number of functional granulocytes (Boxio, Bossenmeyer-Pourie et al. 2004) and the marrow reserve can be mobilised in emergency response to bacterial infections. Numbers of granulocytes reflect bacteremia and sepsis. The high mortality of sepsis has been attributed to an immune overactivation which is linked to a failure of bacterial clearance. The biological mechanisms causing fatal sepsis remain equivocal. Here we analyzed a low-dose infection with Listeria monocytogenes in mice. To mimick immune overactivation, we administered the TLR2/6 ligand Pam2Cys, a Mycoplasma fermentans-derived lipopeptide consisting of palmitoyl side chains acid and a cysteine (Jackson, Lau et al. 2004) or FSL-1, a ligand based on the Mycoplasma salivarium lipoprotein (Shibata, Hasebe et al. 2000). We found that systemic activation of TLR2 by Pam2Cys or FSL-1 resulted in suppression of the bone marrow granulocyte reserve and in uncontrolled bacterial proliferation.

7.3 Material and Methods:

Mice

Specific pathogen free (SPF) laboratory mice were from the institute of Labortierkunde of the veterinary facility of the University of Zurich. Experiments were performed according to Swiss veterinary law and institutional guidelines. C57BL/6 or TLR2-/- mice between 6-10 weeks old were used.

Infections

Listeria monocytogenes (L.m.) strain 10403S grown overnight in Brain Heart infusion broth, washed two times in PBS and frozen at -80°C. Inocula were prepared from thawed aliquots and injected i.v. in 200 μl. Administered lipopeptides were Pam2Cys, a Mycoplasma fermentans-derived lipopeptide consisting of palmitoyl side chains acid and a cysteine, S-(2,3-bis(palmitoyloxy)propyl) cysteine) (Jackson, Lau et al. 2004) and FSL-1, a ligand based on the Mycoplasma salivarium lipoprotein with the formula S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (Shibata, Hasebe et al. 2000).

Bacterial titer

Bacterial titers were determined in homogenized halfs of spleen, the left lobe of the liver, in PBS with serial dilutions plated on brain heart infusion or blood agar plates.

Bone marrow aspirates and culture

Bone marrow cells were flushed with PBS from the femur and were stained for FACS analysis or cultured in RPMI with 10% FCS.

FACS and antibodies

Anti-Ly6G, GR1, CD11b, Annexin V and 7-Amino-Actinomycin-D (7-AAD) were obtained from BD, Basel. Cells expressing Ly6G / GR1 and CD11b are termed “GRC”. FSC/SSC gates were used to exclude debris in organ homogenates and peritoneal washings. Dihydrorhodamine 123 (DHR, Sigma) was used for measuring NADPH oxidase activity by measuring cellular fluorescence in FL1 channel (emission 534nm). A fixed number of fluroescent APC beads were used to quantify cell number per sample volume as has been described before. Anti-GR1 (NimpR14) hybridoma was a generous gift from Dr Tacchini-Cottier, WHO, Geneva.

Histology

Histological samples were snap-frozen in Hanks medium and stained with an anti-L.m. rabbit serum (a gift from Prof J. Bille, Lausanne), Gr-1 (Pharmingen) antibodies. Staining was developed using a goat anti-rat antibody (Caltag Laboratories) or goat anti-rabbit (Jackson Immuno Research) and an alkaline phosphatase-coupled donkey anti-goat antibody (Jackson Immuno Research) with naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid 2-methoxy anilide) phosphate and new fuchsin as a substrate. The presence of alkaline phosphatase activity yielded a red reaction product. The sections were counterstained with hemalum.

Statistical analysis

In all figures, unpaired two-sided t test or one-way analysis of variance (ANOVA) was used. P values above 0.05 were not considered significant. * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$.

End notes:

A.A. Navarini and K. Lang have contributed equally to this work.

7.4 Results

To evaluate bone marrow granulocytes during TLR2/6 stimulation, we administered 100 µg of the lipopeptide Pam2Cys intravenously in C57BL/6 mice. After 24 hours, we investigated the number of granulocytes within the bone marrow (Fig. 1a). Surprisingly, granulocytes ($Ly6G^+CD11b^+$) were reduced by 90-95% numerically and by percentage (Fig. 1b). This reduction could have resulted from two effects – on one hand, granulocytes leaving the bone marrow, on the other hand, granulocyte death within the bone marrow. When the percentage of granulocytes that had entered apoptosis after Pam2Cys treatment was measured, up to 30% of granulocytes were apoptotic compared to 10% in controls (Fig. 1c). $TLR2^{-/-}$ granulocytes instead were almost completely resistant to this stimulus, proving that TLR2 engagement was required for the observed granulocyte loss. The strong reduction and apoptosis was specific for granulocytes as monocytic precursors that were $CD11b^+$ and M-$CSF(CD115)^+$ were not reduced by TLR2/6 ligation (Supp. Fig. 1a). To determine whether Pam2Cys also provoked granulocyte emigration from the bone marrow, the number of granulocytes in the spleen (Fig. 1d) and liver (Supp. Fig 1b) was measured. We found that Pam2Cys treatment resulted in great accumulation of apoptotic, but not viable granulocytes in the spleen (Suratt, Young et al. 2001). In the liver, no increase of granulocytes could be demonstrated. Therefore, Pam2Cys induced bone marrow apoptosis and emigration of pre- and fully apoptotic granulocytes. In the blood, no significant increase of granulocytes could be measured (Supp. Fig. 1c).

We wondered whether the observed granulocyte death was associated with activation. When an early activation marker, the β_2-integrin CD11b (Witthaut, Farhood et al. 1994) was tested, a clear difference between Pam2Cys treated and control granulocytes was observed (Fig. 1e). $TLR2^{-/-}$ bone marrow granulocytes showed elevated CD11b expression compared to controls in the naïve state, however this was not further increased by Pam2Cys treatment. When granulocytes are fully activated, they activate the destructive respiratory burst machinery that eventually results in their own cell death (Watson, Redmond et al. 1996; Engelich, White et al. 2002). To analyze granulocyte oxidative burst, the mean fluorescence of granulocytes after incubation with Dihydrorhodamine 123 was measured. TLR2 ligation by Pam2Cys resulted in specific activation of the respiratory burst (Fig. 1f).

To determine whether these effects could be re-produced by direct incubation of

bone marrow with TLR2/6 ligands, Pam2Cys (or FSL-1, not shown but similar results) were added in increasing amounts to C57BL/6 bone marrow eluates. 24 hours later, activation and apoptosis was measured. Both reagents induced activation starting from 1ng/ml (Fig. 2a,b,c), with full- blown activation at 10 and 100 µg/ml. Reaction to Pam2Cys *in vitro* was mainly TLR2-dependent, however, with the highest dose, also some reaction by TLR2-/- bone marrow was detected, which might be possibly mediated by TLR6 known to also interact with lipopeptides (Takeuchi, Sato et al. 2002). Therefore, systemic circulation of soluble TLR2 ligands resulted in activation and depletion of the bone marrow granulocyte reserve. As during bacterial infections such as listeriosis, contact of bacterial products can occur with the blood stream, we wondered whether a bout of circulating bacterial products could therefore significantly influence the prognosis of a low-level infection.

Therefore, C57BL/6 mice or TLR2-/- controls were infected with 1000 cfu L.m. intravenously (Fig. 3a). This dose is sublethal in C57BL/6 mice, 1/10 LD_{50}. At day 1 of infection, the TLR2/6 ligand Pam2Cys was administered in a dose of 100 µg. 2 days later, at day 3 of the bacterial infection, the bacterial titers were measured (Fig. 3b). The Pam2Cys treated animals' organs showed 10 to 100 fold higher bacterial titers than controls. TLR2-/- animals were resistant to the effects of Pam2Cys. As comparison for complete absence of granulocytic activity, a granulocyte depleting antibody (NimpR14, anti-GR1) was used. 100 µg anti-GR1 were given on day 1 of the L.m. infection, which similarly lead to increased bacterial titers. To determine whether this was associated with decreased supply of granulocytes, we measured numbers of $Ly6G^+CD11b^+$ cells in the blood (not shown) and bone marrow (Fig. 3c). Both in blood and in bone marrow, granulocytes were strongly depleted in the Pam2Cys treated animals. TLR2-/- mice treated with Pam2Cys or PBS at day 1 of the infection survived the infection, as did PBS-treated wildtype mice. Pam2Cys-treated wildtype mice however developed clinical disease at day 4-5.

By immunohistochemistry, granulocyte-dependent containment of L.m. was investigated in the liver (Fig. 3e). Untreated animals harboured few bacteria, which were densely packed with infiltrating granulocytes at day 3 of the infection. The Pam2Cys treated animals showed multiple liver foci consisting of bacterial lesions, whereas infiltrating granulocytes were almost completely lacking. Taken together, Pam2Cys treated animals were highly susceptible to Listeria which correlated with granulocytopenia in bone marrow and target organs.

7.5 Discussion

Here we report that TLR2/6 ligands reaching the systemic circulation activated and destroyed the bone marrow granulocyte supply. When this occurred during a non-lethal bacterial infection, decreased containment of bacteria was found, leading to lowered survival.

In clinical medicine, it is well known that granulocytes are essential for overcoming bacterial infections (Watts 1999). One of the important predictors of susceptibility to bacterial infection is neutropenia and agranulocytosis (Finberg and Talcott 1999). Neutropenia is for example observed in cancer therapy, as part of haematologic diseases. Importantly, administration of type I interferon is a known cause for neutropenia in humans and in mice, underlining that innate immune activation may be detrimental for granulocytes (Navarini, Recher et al. 2006). Since 30 years, many studies with granulocyte transfusions have been attempted to achieve protection during bacterial infections in neutropenic patients (Graw, Herzig et al. 1972; Herzig, Herzig et al. 1977; Stanworth, Massey et al. 2005), however with conflicting results. Possibly, the transfused peripheral blood granulocytes had too short a half-life. Today, with advanced cell culture techniques, it might be possible to transfuse earlier maturation states of granulopoiesis that might form a better reservoir. In our hands we have observed that transfused bone marrow granulocytes homed efficiently to the bone marrow, but only when no bacterial infection was already active in the recipient (manuscript in preparation). Nevertheless, the amplification of granulopoesis by administration of GM-CSF is already commonly used to shorten the duration of neutropenia following cancer treatment.

Sepsis means a bacterial infection combined with a systemic inflammatory reaction syndrome (SIRS) (Muckart and Bhagwanjee 1997). It is defined by presence of >12000 or <4000 leukocytes per μl blood (which are mainly granulocytes in humans), and reactions of the heart (tachycardia), lungs (hypoxaemia) and the brain (fever or hypothermia). Sepsis has a high mortality. Since high cytokine concentrations are linked to a poor outcome (Osuchowski, Welch et al. 2006), it is generally believed that lethal sepsis is linked to immune overactivation. However, immunmodulating therapies such as anti TNF antibodies (Abraham, Wunderink et al. 1995) alone have failed so far. Our results might implicate that immune overactivation during sepsis might reduce the available granulocyte pool in the bone marrow and thereby reduce the antibacterial immune response.

At the dosages used, Pam2Cys did not induce a shock-like syndrome when administered to mice – on the contrary, mice remained completely symptom-free. Interestingly, similar dosages are used regularly for priming of T cells (Zhang, Issagholian et al. 2005).

What other stimuli can lead to the observed effects on granulocytes? In this study, we tested only TLR2 ligands. However, to our opinion it is likely that also during gram-negative infection, stimulatory molecules such as LPS act on the bone marrow as resistance-lowering agents. Interestingly, after activation by Pam2Cys and during sepsis, macrophage numbers are not suppressed (Supp Fig. 1b) and (Ellaban, Bolgos et al. 2004). As during sepsis, TNF-α has been implicated to result in immunosuppression, we have tested TNF receptor 1 -/- mice, which showed similar bone marrow granulocyte suppression as wildtype mice (not shown). As we had identified a proapoptotic effect on bone marrow granulocytes by IFN I (chapter 6), we treated also IFNAR-/- as well as IFNG-/- mice with Pam2Cys, which however resulted in similar bone marrow granulocyte apoptosis as wildtype controls (not shown).

Taken together, our results demonstrate that during an innocuous bacterial infection, an additional rather weak (non-toxic) immunstimulation via TLR engagement may have fatal consequences. Our results show that immune overactivation may be linked with granulocyte apoptosis within the bone marrow and may help to explain sepsis.

7.6 Author contributions

A. Navarini performed the greater part of the experiments and wrote the manuscript. K. Lang performed the smaller part of the experiments and gave intellectual help. Assistance with experiments as well as intellectual help was provided by M. Recher and A. Verschoor. Histology was performed by Prof. B. Odermatt. The work was performed in the laboratory of Prof. R.M. Zinkernagel and Prof. H. Hengartner who guided the work and manuscript.

7.7 Figure legends:

Fig. 1: Decreased granulocyte numbers in bone marrow, enhanced activation-associated apoptosis and sequestration of apoptotic granulocytes in the spleen after Pam2Cys administration.

(A) Bone marrow granulocytes (GRC, $Ly6G^+CD11b^+$) cell numbers or (B) percentages measured by FACS 24 hours after administration of 100 µg Pam2Cys i.v. (Mean +/- SEM, n=3-5 animals per group, one of two experiments with similar outcome). (C) Percentage of apoptotic ($AnnexinV^+7AAD^+$) GRC in bone marrow 24 hours after 100 µg Pam2Cys i.v. (Mean +/- SEM, n=3-5 animals per group, one of two experiments with similar outcome). (D) Number of granulocytes in spleens 24 hours after administration of 100 µg Pam2Cys, black bars show non-apoptotic (7AAD-AnnexinV-) granulocytes, red bars show $AnnexinV^+7AAD^+$/- GRC (Mean +/- SEM, n=3-5 animals per group, one of two experiments with similar outcome). (E) Mean fluorescence intensity of CD11b surface staining on bone marrow GRC 24 hours after administration of 100 µg Pam2Cys i.v. (Mean +/- SEM, n=3-5 animals per group, one of two experiments with similar outcome). (F) Mean fluorescence intensity of DHR123 staining in bone marrow GRC 24 hours after administration of 100 µg Pam2Cys i.v. (Mean +/- SEM, n=3-5 animals per group, one of two experiments with similar outcome).

Fig. 2: Pam2Cys treatment of bone marrow in vitro results in dose-dependent activation and cell death

(A) Mean fluorescence intensity of DHR123 staining in bone marrow granulocytes incubated for 24 hours with different amounts of Pam2Cys (Mean +/- SEM, n=4, one experiment) (B) Mean fluorescence intensity of CD11b staining on bone marrow granulocytes incubated for 24 hours with different amounts of Pam2Cys (Mean +/- SEM, n=4, one experiment). (C) Percentage of dead ($7AAD^+$) bone marrow granulocytes after 24 hours of incubation with different amounts of Pam2Cys (Mean +/- SEM, n=4, one experiment).

Fig. 3: Systemic TLR2 ligation during non-lethal bacterial infection leads to absence of abscess-associated granulocytes and uncontrolled bacterial propagation

(A) Experimental protocol (B) Survival curve of TLR2-/- or wildtype mice treated with 100µg Pam2Cys or PBS at day 1 of infection with 1000 cfu Listeria monocytogenes (n=5-11 per group). (C) Bacterial organ titers at day 3 of infection with 1000 cfu Listeria monocytogenes with or without administration of 100 µg Pam2Cys 24 hours after infection. Green bars show C57BL/6 controls that had received 100 µg anti-GR1 antibody at 24 hours after infection. (Mean +/- SEM, n=3, one of two experiments with similar outcome). (D) Bone marrow granulocytes at day 3 of the listerial infection. (Mean +/- SEM, n=3, one of two experiments with similar outcome). (E) Immunohistochemistry of livers at day 3 of listerial infection stained for GR1 (GRC) or anti-L.m. serum. Injection of PBS at day 1 shown on the left, administration of 100 µg Pam2Cys at day 1 to the right.

Supplementary Figure 1:

(A) Pam2Cys administration does not suppress bone marrow monocytes: Numbers of M-CSF-R^+($CD115^+$)$CD11b^+$ cells in bone marrow 24 hours after administration of 100 µg Pam2Cys i.v.

(B) Granulocyte sequestration does not take place in the liver: Number of granulocytes in livers 24 hours after administration of 100 µg Pam2Cys, black bars show non-apoptotic (7AAD-AnnexinV-) granulocytes, red bars show $AnnexinV^+7AAD^+$/- GRC (Mean +/- SEM, n=3 animals per group, one experiment).

(C) Blood granulocytes are not significantly suppressed after administration of Pam2Cys: Numbers of granulocytes in blood 24 hours after administration of 100 µg Pam2Cys i.v.

7.8 References:

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Fig. 1: Pam-2-Cys leads to decreased granulocyte numbers in bone marrow, by activation-associated apoptosis and sequestration of apoptotic granulocytes in the spleen

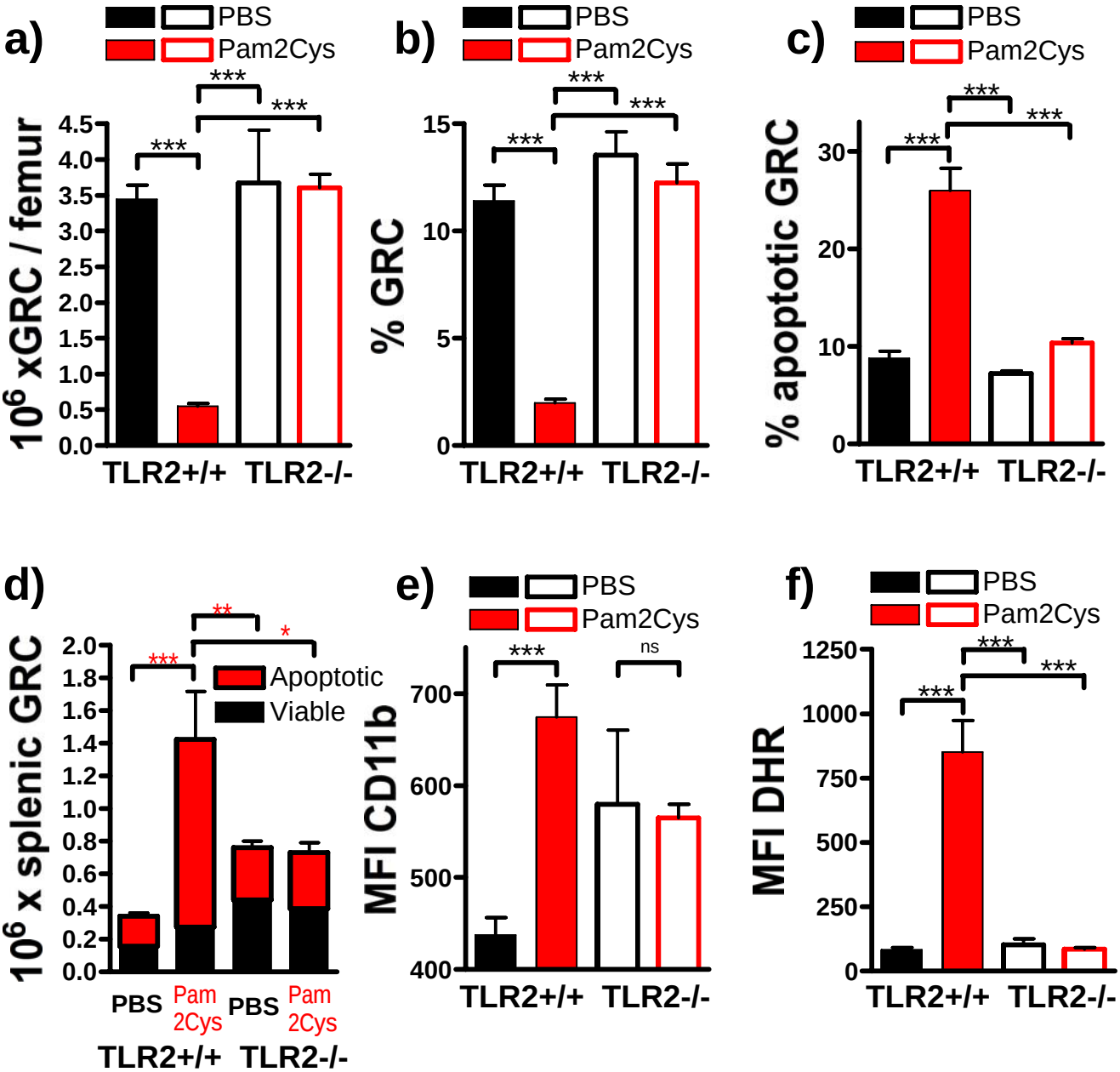


Fig. 2: Pam-2-Cys treatment of bone marrow cells *in vitro* results in dose-dependent activation and cell death

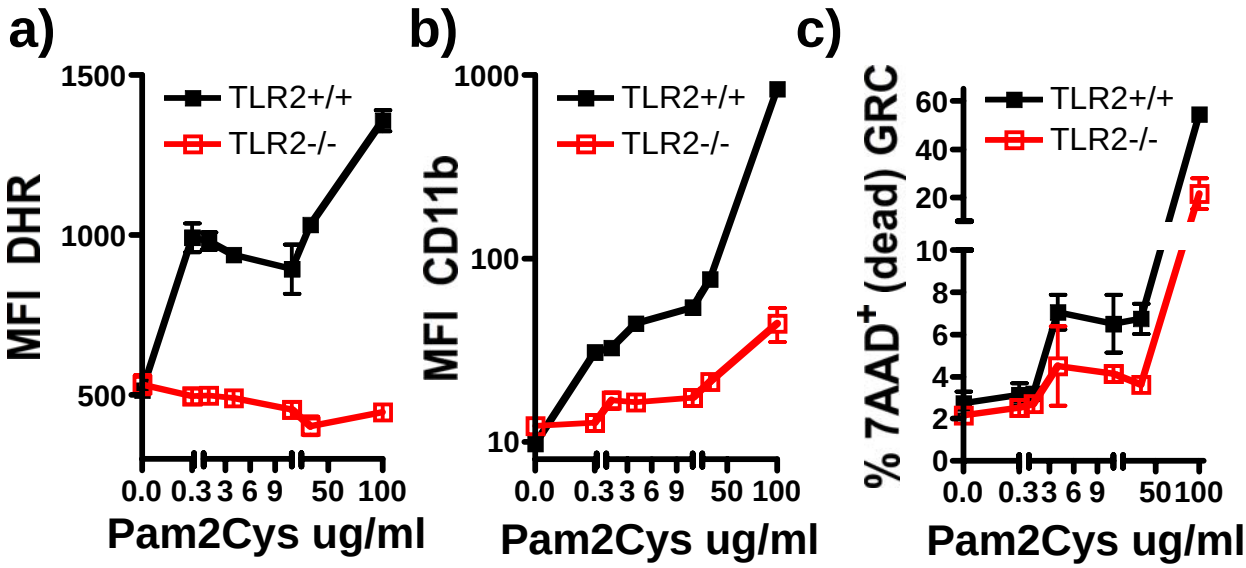
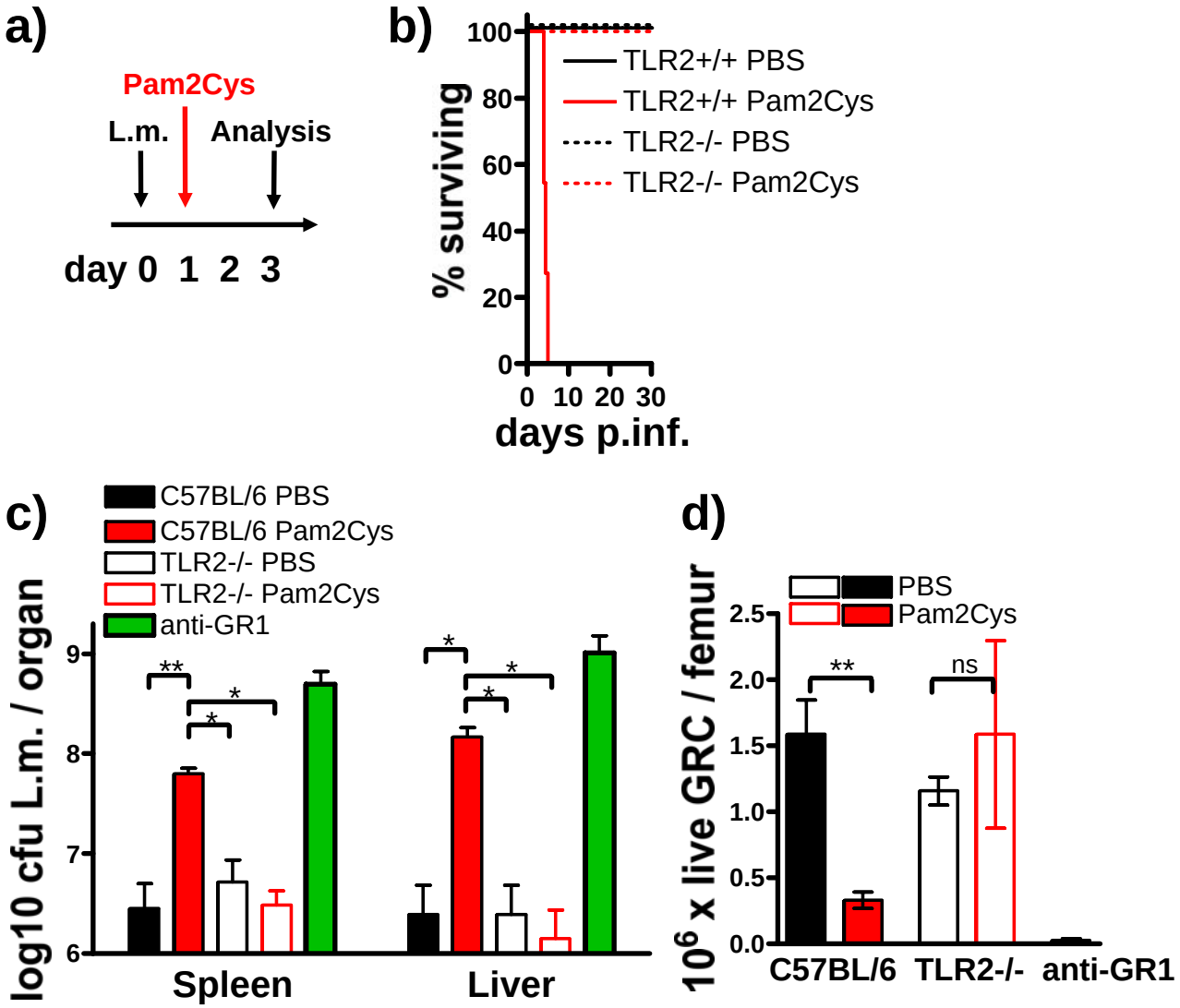


Fig. 3: Systemic TLR2 ligation leads to absence of abscess-associated granulocytes, uncontrolled bacterial propagation and host death



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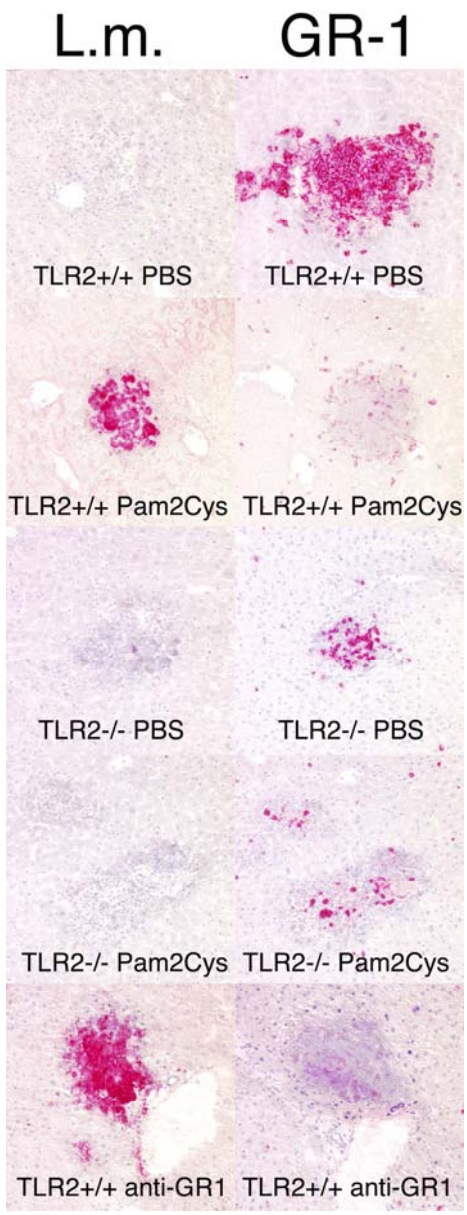
L.m.
GR-1
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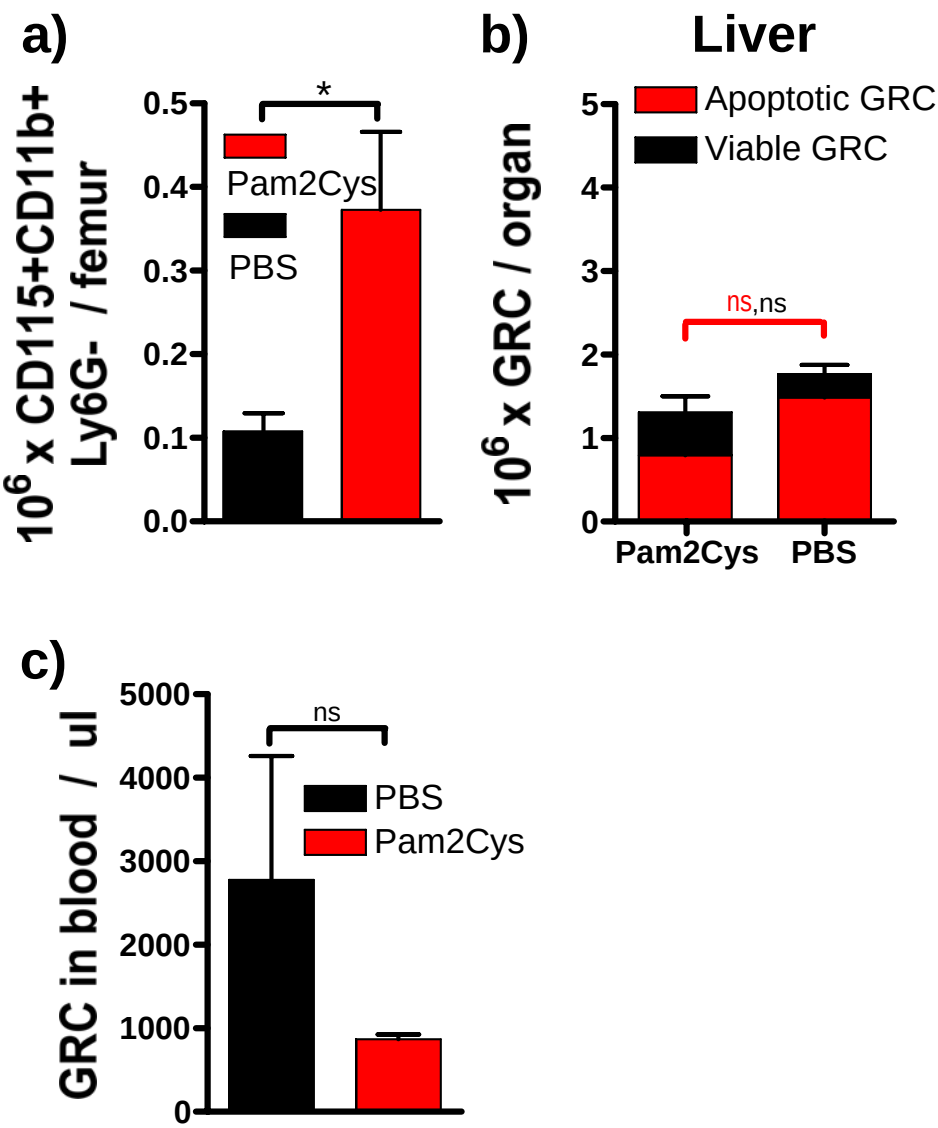
TLR2-/- Pam2Cys
TLR2-/- Pam2Cys
TLR2+/+ anti-GR1
TLR2+/+ anti-GR1

Supplementary Figure 1:
a) Pam-2-Cys administration does not suppress bone marrow monocytes
b) Granulocyte sequestration does not take place in the liver
c) Blood granulocytes are not significantly suppressed after administration of Pam-2-Cys



8. TLR4 ligation results in prolonged innate resistance to bacterial challenge

8.1 Abstract

Antigen-specific immunity greatly increases survival of reinfections. Here we evaluated how administration of lipopolysaccharide as an antigen-unrelated stimulus resulted in increased resistance to the grampositive bacterium Listeria monocytogenes. Prolonged "innate immunity" was short lived, unspecific and mainly resulted from induction of granulocyte numbers in the bone marrow. Short term, unspecific innate resistance effects by past events may have great significance in host survival, particularly when living in pathogen-rich environments.

8.2 Introduction

An organism challenged with a sublethal infection usually develops strong adaptive immunity against the pathogen, and therefore can specifically react against a second challenge. This often results in heightened chances of survival of a second infection. Maintenance of sufficiently high neutralizing antibody titers correlates with protection against acute cytopathic infections. Maternal antibodies are transmitted to offspring and play an important role in early survival (Zinkernagel 2001). Because adaptive immune responses are antigen-specific, the first encounter of a new infection induces a primary adaptive response that takes several days to develop.

Thus, the subject may at first be initially unprotected by adaptive memory. In addition to adaptive immunity, innate resistance mechanisms, particularly by granulocytes and macrophages, play an important role in resistance to infections (Janeway and Medzhitov 2002). To date, it is unclear whether past activation of the innate immune system can produce lasting effects and for how long, whether this can contribute to resistance against pathogens, and which cells mediate such effects. Several studies have reported unspecific increased resistance against pathogens after prior stimulation by activating agents that cannot be readily explained by adaptive memory (Medina, Vas et al. 1975; Uchiya and Sugihara 1989). Instead, the concept of induced unspecific activation was used to explain resistance against many pathogens after an experimentally defined single stimulation. This resistance was described as lasting from 24 hours (Noursadeghi, Bickerstaff et al. 2002) up to 60 days (Baughn and Bonventre 1975; Medina, Vas et al. 1975). While adaptive immunity is often protective for months to years, the wide spectrum of innate resistance renders understanding of memory-like aspects of innate immunity potentially interesting.

Here we evaluated whether hosts that recently had encountered an innate activating stimulus would fare better against a new pathogen and if so, for how long this could such preparedness could last.

These questions were evaluated by stimulation of the innate immune system with the TLR4 ligand lipopolysaccharide. Changes in the respective innate cell types were investigated by histology and FACS. Protection against a challenge infection was assessed with Listeria monocytogenes (L.m.) infection. This gram-positive, lipopolysaccharide-negative bacterium initially targets liver and spleen and replicates intra- and extracellularly. The host response has two phases, first mediated

predominantly by granulocytes, the later phase initiated by day 3 is mediated by CD8+ and CD4+ T lymphocyte-induced macrophage activation.

We show that an innate activation state can be measured by elevation of granulocyte numbers in bone marrow and CD11b upregulation. Our experiments here show that TLR4 ligand administration caused granulocyte accumulation and activation, that correlated with increased survival.

8.3 Material and Methods:

Mice

Specific pathogen free (SPF) C57BL/6 mice (6-10 weeks old) were from the institute of Labortierkunde, University of Zurich. Experiments were performed according to Swiss ethical protection laws and institutional guidelines.

Infections

Listeria monocytogenes (L.m.) strain 10403S were grown overnight in Brain Heart infusion broth, washed two times in PBS and frozen at -80°C. Inocula were prepared from thawed aliquots and injected i.v. in 200 μl.

Bacterial titer

Bacterial titers were determined in homogenized ½ spleen and of the left lobe of the liver. Serial dilutions in PBS were plated on brain heart infusion plates.

Bone marrow aspirates and culture

Bone marrow cells were flushed with PBS from the femur and were stained for FACS analysis or cultured in RPMI with 10% FCS.

FACS and antibodies

Anti-Ly6G, GR1, CD11b, Annexin V and 7-Amino-Actinomycin-D (7-AAD) were obtained from BD, Basel. Cells expressing Ly6G / GR1 and CD11b are termed “GRC”. FSC/SSC gates were used to exclude debris in organ homogenates. A fixed number of fluorescent APC beads were used to quantify cell number per sample volume as has been described before (REF). Anti-GR1 (NimpR14) hybridoma was a generous gift from Dr. Tacchini-Cottier, WHO, Geneva.

Statistical analysis

In all figures, unpaired two-sided t test or one-way analysis of variance (ANOVA) was used. P values above 0.05 were not considered significant. * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$.

8.4 Results

C57BL/6 mice were treated with 300μg LPS i.v. and analysed during 14 days. To assess changes in the innate immune system, phagocyte numbers were measured as follows. Granulocyte numbers in bone marrow eluates were measured by FACS for $Ly6G^+CD11b^+$ cell count at different timepoints after i.v. administration of 300μg LPS. At 24 hours, a drastic reduction of granulocyte numbers was found in the bone marrow. However, granulocyte numbers had recovered by day 5, leading to an increase of up to 200% of the original numbers by day 7 and 9, thereafter they decreased during the following 5 days (Fig. 1a). At day 7 and 9, the remaining (Ly6G-negative CD11b-negative) bone marrow cells were reduced in number (Fig. 1a). When blood granulocytes were investigated, we found that numbers were within normal ranges at 24 hours. By day 7 they had increased corresponding to the reserve in the bone marrow. Monocytes also arise from the bone marrow. Measurement of the monocytes by defined by $Ly6C^+CD11b^+Ly6G^-$ staining in bone marrow eluates at the different timepoints after LPS administration revealed no drastic variation in numbers (Fig. 1a).

In addition to monocytes and granulocytes, fixed macrophages are the remaining important phagocytic compartment that contributes to antibacterial defense. To determine whether their activation status after LPS treatment, their activity was assessed by the activation markers CD80, CD86 and MHC II (I-Ab) in the spleen and liver (Fig. 1b). Surprisingly, all three markers showed a trend to downregulation at day 7 after LPS treatment. Immunohistochemistry showed that tissue macrophages were present during the whole period until day 14 (not shown).

Taken together, LPS treatment influenced predominantly granulocyte numbers in the bone marrow. This treatment also altered their bactericidal activity, assessed with the model infection of mice with *Listeria monocytogenes*. These gram-positive bacteria target the liver and spleen and replicate intra- and extracellularly. The host response has two phases, the first being mediated predominantly by granulocytes, the later phase initiated by day 4 is mediated by $CD8^+$ and $CD4^+$ T lymphocyte-induced macrophage activation.

C57BL/6 mice were treated with 300μg LPS, and infected with 10'000 cfu L.m. 7 days later (Fig. 1c). At day 3 of the infection, bacterial titers were determined in the spleen and liver (Fig. 1d). Both organs of LPS-pretreated animals had 5-20 fold lower bacterial colonies than PBS-pretreated controls. When in addition on day 1 of the

bacterial infection, 100 μg of the granulocyte-depleting antibody anti-GR1 was administrated, the titer difference was abrogated, indicating that presence and efficiency of granulocytes correlated with the LPS-induced antibacterial resistance. LPS-pretreated mice survived better than PBS-treated controls after L.m. infection with 3-5 LD^{50} (Fig. 1e). LPS- or PBS-pretreated C57BL/6 mice were given $3x10^4$ cfu or $5x10^4$ cfu L.m. at day 7 and monitored for signs of disease. Control mice all succumbed at day 3-4 to the infection, while LPS-pretreated animals showed 75% survival to $3x10^4$ cfu and 25% to $5x10^4$ cfu L.m. Taken together, our results demonstrated increased anti-bacterial resistance induced by administration of lipopolysaccharide. This resistance correlated with bone marrow granulocytosis and was abrogated by granulocyte depletion.

8.5 Discussion

The results showed that administration of the TLR4 ligand lipopolysaccharide induced elevation of granulocyte numbers in bone marrow. These changes peaked after 7 days and waned after 15 days. A bacterial infection during this period was controlled by early bacterial clearance and resulted in increased survival of LPS-pretreated mice.

This “memory” like prolonged resistance is granulocyte dependent and lasts at least for some 7 days. T cell dependent macrophage activation is often dependent on persisting antigen by intracellular bacteria or virus antigens and could last long, while the innate resistance-memory is of limited duration. What is not clear from our experiments, is whether granulocytes from LPS-treated mice show higher bactericidal activity. Interestingly, it has been shown that even when mice are immunized against L.m., the killing activity of early granulocytes does not differ from that of naïve controls. Only the number of phagocytes that can be recruited is much higher in immune mice (Czuprynski, Henson et al. 1984; Czuprynski, Henson et al. 1985). Taken together, our observation suggest an addition and widening of the “hygiene hypothesis”: LPS and probably other TLR ligands can activate innate immunity for 5-7 days, enhancing protection against bacterial superinfection.

8.6 Author contributions

A. Navarini performed the greater part of experiments and wrote the manuscript. The smaller part of the experiments was performed by A. Verschoor who also assisted with the manuscript. Help with experiments as well as intellectual help was provided by K. Lang and M. Recher. Histology was performed by Prof. B. Odermatt. The work was performed in the laboratory of Prof. R.M. Zinkernagel and Prof. H. Hengartner who guided the work and manuscript.

8.7 Figure legends

Fig. 1: LPS induces granulocyte-dependent antibacterial resistance (A) Numbers of $Ly6G^+CD11b^+$ (red), $Ly6C^+CD11b^+Ly6G$- (black), and Ly6G-CD11b- cells (green) after administration of 300μg LPS i.v. (n=3-4 per timepoint). (B) Mean fluorescence intensity of I-Ab, CD80 and CD86 on live $F4/80^+$ cells at day 7 after administration of LPS (Mean +/- SEM, n=4-5, one of two experiments). (C) Experimental protocol (D) Bacterial organ titers at day 3 of infection with 10^4 cfu L.m. monocytogenes with or without administration of 100 µg anti-GR1 i.v. 24 hours after infection. (Mean +/- SEM, n=3). (E) Survival curve of mice infected with $3x10^4$ or $5x10^4$ L.m. 7 days after LPS or PBS treatment (n=4 per group, one of two experiments).

8.8 References:

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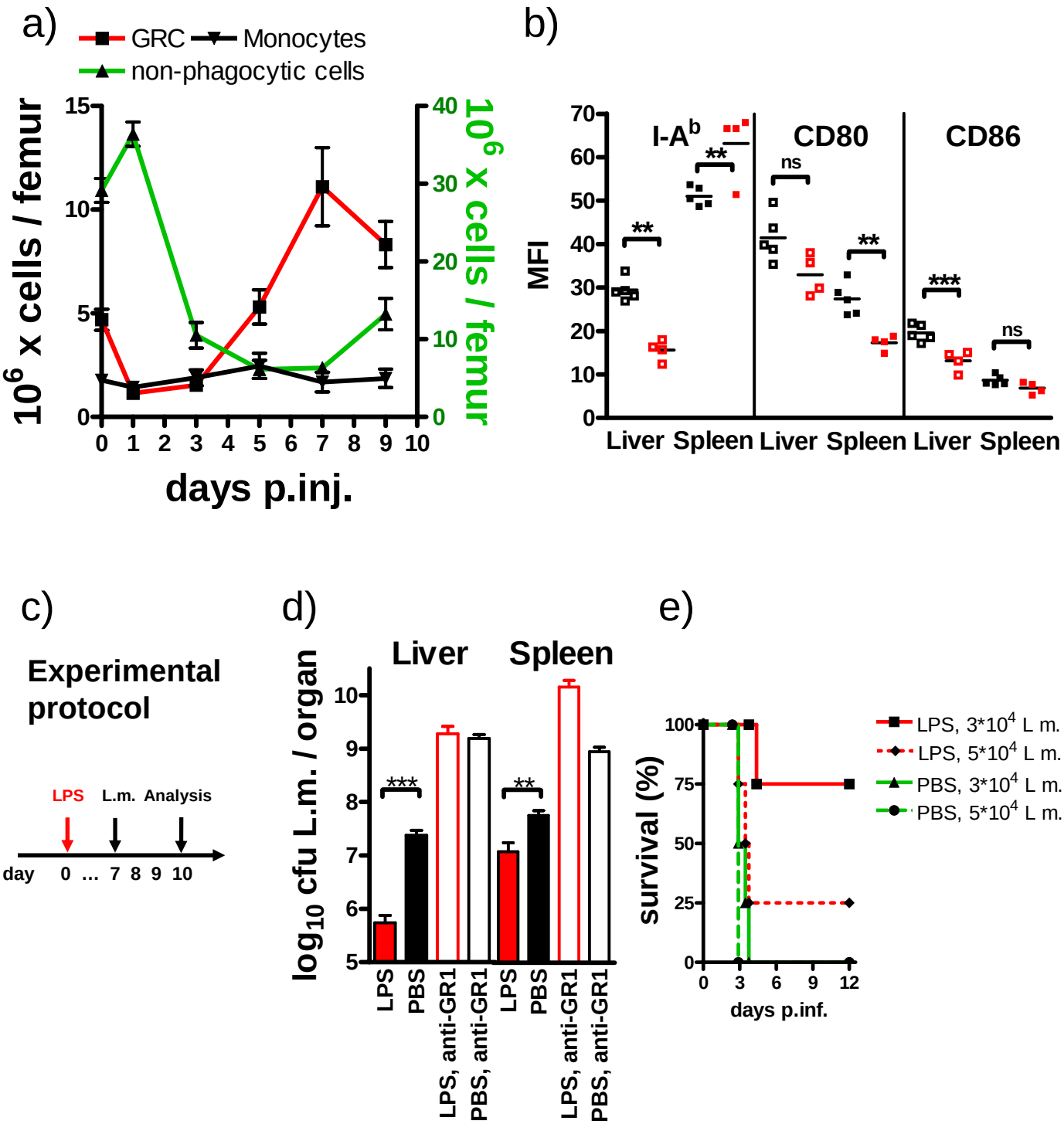
Fig. 1: LPS induces granulocyte-dependent antibacterial resistance



9. Discussion

We have analysed granulocyte kinetics in four different situations. First, a bacterial infection was administrated and analysed. Next, a virus infection was given prior to the bacterial infection. As third approach, systemic administration of a TLR2 ligand and its effects on a bacterial infection was analysed. Lastly, we analysed the effects of TLR4 stimulation prior to bacterial infection. Taken together, we observed that changes in the bone marrow granulocyte reserve influenced bacteria clearance.
To put these findings into some perspectives, I would like to pose some general questions about immunology and later in the chapter discuss implications of the different findings.

1. How can a host best inhibit an unknown infectious pathogen?
2. How can the host protect its offspring during the early phase of immunoincompetence?

The answer to the first question is to react with speed and a low threshold to any foreign antigen that can easily be recognised as such. This detection must be provided by receptors that are constitutively expressed on many cells, so that a quick reaction can result. Receptors that fulfill this requirement are the pattern recognition receptors. Their expression is widespread and they are non-clonal (Muzio, Bosisio et al. 2000; Janeway and Medzhitov 2002). As soon as a clearly foreign molecule, i.e. a bacterial lipopeptide or lipopolysaccharide that is not expressed by the host binds to such a receptor, the immune system can be safely activated without danger of autoimmunity. Clonal receptors such as the T or B cell receptor are not useful for this purpose as in the naïve state, only a few of these express the same receptor.

Speed and low threshold of reactivity

Why are speed and a low threshold probably very important? If one looks at the problem from the pathogen's point of view, its main goal is to quickly establish a persistent infection in the host. This must be done without being noticed for as long as possible to multiply and invade the host's tissues, so that the chances of being cleared decrease over time. Because pathogens also undergo evolution, the respective pathogens abilities to achieve a quick and stealthy infection were selected

for during the co-existence of host and parasites. Therefore, during evolution of pathogens, they will be selected for low triggering of pattern recognition receptors and for quick establishment of a persistent infection. These selected abilities need to be countered by a low threshold of activation and quick reaction by the host if he is to survive.

A constant supply of mobile phagocytes must be maintained

What are the implications of a low threshold for activation? If the host has to provide a quick immunological reaction to every bacterial infection, a constant arsenal of antibacterial mediators must be at ready disposition. Pathogens not previously encountered cannot be instantly countered with reactions by B cells or T cells. Therefore, a high number of ready-to-go phagocytes are needed to react quickly to any foreign antigen. This system of mobile phagocytes has such a low threshold of reaction that activated granulocytes infiltrate target organs very quickly (within 12 hours) even if i.e. only few bacteria reach the liver.

High reactivity can result in vulnerability to overactivation

However, if we assume that a high reactivity to foreign antigens is indeed beneficial, what are the disadvantages? If a system is fine-tuned for reactions to low antigen loads, it does not necessarily mean that it will be able to compensate sudden high amounts of antigen. For example, if the granulocytes have a low threshold of reacting against lipopeptides because they are anticipating bacterial infection, then what would happen if such a lipopeptide entered the systemic circulation? Quite possibly, the granulocytes would be activated all at once - and this is what we observed in chapter 7 when the TLR2 ligand Pam2Cys was administered intravenously. That a generalized activation of granulocytes could be a common and dangerous situation becomes clear if one thinks about the phenomenon of sepsis, which is defined as bacteria in the circulation eliciting a systemic response. Surprisingly, the susceptibility to a bacterial infection was greatly increased after administration of Pam2Cys. As explanation, we found that not only were granulocytes activated, in fact there were almost no granulocytes left in the bone marrow to supply the active abscesses in the liver. In humans during sepsis / SIRS two phases can be distinguished, defined by leukocytes above 12000 or below 4000 per μl blood. Therefore, the systemic response by circulating bacteria or bacterial products can have differential effects on leukocyte counts.

Therefore, the price for high reactivity to a low antigen load might be that overactivation can result quite easily and lead to temporary exhaustion of the response. If the bone marrow granulocytes were harder to activate, perhaps the chances would be that a dangerous peripheral bacterial infection were ignored until too late?

Latency to downregulate innate activation can be advantageous

To have continued activation of the antibacterial mechanisms after clearing the infection could be an additional mechanism to help overcome infections. If the host has been infected with an unknown pathogen in his habitat, right after the infection the chances could be higher to encounter a second infection either from the same or even another pathogen. It is known that new infectious diseases arise during habitat changes either from the habitats side such as temperature, weather, migrating animals (Shope 1991), or from the hosts side such as migration (Diamond 1997). It is therefore feasible that after clearance of an infection, it were of some advantage if the host's defence remained activated for a while.

There are two possibilities to do this. The first would be to leave phagocytes increased in numbers and reactivity. Evidence for such a relatively short-lived mechanism was found when Lipopolysaccharide was administrated and granulocyte numbers counted later 7 days later (chapter 8). Such unspecific mechanisms could have the advantage that the defence could be increased against every infection that can be recognized by pattern recognition receptors expressed on granulocytes.

Maintenance of immune responses uses energy – innate more than adaptive

Any bacterial or even viral infection could possibly be stopped better if phagocytes were increased, but only with some costs to the host. Although plants do not have mobile phagocytes, it has been known for a long time that in plants, mechanisms exists that result in generalized resistance (van Loon 1977; Kachroo, Chandra-Shekara et al. 2006). However, when one experimentally tried to induce this mechanism constitutively in harvest plants, it was found that they did not grow well anymore, apparently having invested too much energy in the defence mechanism (Kaniewski and Thomas 1999). The same could theoretically be true for having granulocyte numbers constitutively elevated and/or activated.

The second mechanism of lasting protection is well known – it is to prime memory T or B cells and keep them stored until a second infection results. This mechanism has the advantage that probably only little energy has to be invested. The disadvantage

is that the memory T cells are antigen-specific and cannot be used against a new infection. However, T cell dependent macrophages represent longer term effective “unspecific” resistance mechanisms. The same is true for having specific B cells that secrete antibodies – they work only against known infections. However, both T and B cells are very energy-efficient and have therefore possibly evolved out of more non-specific general but energy-wasting defence mechanisms (Li, Barreda et al. 2006). A host without B or T cells in an environment where he repeatedly encounters the same infection, he would have to constantly undergo full-fledged granulocyte/macrophage activation if he did not have T and B cells. With an adaptive immune system, only the specific T cells and a few phagocytes need to be activated to control a second infection.

It has been debated why memory T cells should be necessary (Zinkernagel 2003). When the organism has survived an infection the first time, his chances are very high to survive it a second time – therefore, T cell memory is possibly not crucial for the survival of the individual, at least until reproduction. However, one may speculate be that energy consumption makes a crucial difference – if an organism has T cell memory, he does not need full-fledged granulocyte/macrophage activation every time a pathogen is encountered anew, but only a specific adaptive immune response. Therefore, more energy remains for reproduction and raising offspring.

Maternal antibodies offer transmissible protection

So what could be the answer to the second question - how to best to protect offspring? It has been observed that calves having received no colostrum after birth died from many infections. It has later been found that maternal antibodies produced by the mother are transmitted to the offspring transplacentally and by mothers milk (Gitlin, Hitzig et al. 1956; Brambell 1966; Plotkin 2001). These antibodies reflect the spectrum of antigens that the mother has encountered up to pregnancy. If the offspring receive these antibodies, a temporary protection ensues that lasts for 6-24 months in humans and much shorter in mice. However, after this period, due to the 20 day half-life of the antibodies, the antibody titers drop below protective values.

Mothers milk can turn deadly infections in vaccinations

Thereafter, the offspring have to depend on their own immune system and undergo all the infections anew. Interestingly, maternal antibodies can attenuate all antibody-susceptible infectious diseases during the first months of life – but usually do not lead to total inactivation of the pathogens. This could be an appropriate setting for developing the offsprings own immunity against a pathogen. It could mean that the offspring could benefit from other members of the population suffering from a contagious infectious disease because with frequent contacts, the offspring could be effectively vaccinated. If none of these pathogens were circulating in the population, they would not have the chance to encounter it during the period of protection and would not develop protective immunity. In this manner, the population as a whole could profit from having some members which are weak and still harbour infectious agents. We have performed experiments that involved exposure of maternally protected pups to otherwise lethal virus infections (see Chapter 12) and found that long-lasting immunity resulted.

Interferon type I and bone marrow granulocytes

We observed that IFN I action can reduce granulocyte numbers and facilitate superinfection. If these results are generalized, it could mean that during every antiviral response with high IFN I levels, granulocytopenia results in the bone marrow. How are these negative effects on granulocytes mediated? The main effect of IFN alpha and beta has been shown to be inhibition of virus transcription, activating RNA degradation, upregulating MHC class I and slowing or stopping the cell cycle (Voutsadakis 2000; Caraglia, Vitale et al. 2004; Kuchtey, Chefalo et al. 2005). Further, it is known that IFN I can be used to treat hairy cell leukaemia effectively, where it induces apoptosis (Baker, Pettitt et al. 2002). Therefore, it could be that the granulotoxic effects we observed could be due to similar mechanisms as apoptosis induced in hairy cell leukaemia cells by IFN I.

Biological role of granulotoxic effect of IFN I is unclear

It would be interesting to know whether apoptosis induction in bone marrow granulocytes by IFN I has a biological reason or not. It is not clear from our results whether the granulocytes are directly targeted by IFN I or whether this reflects only an extreme situation. Further, it remains unknown whether this effect has been selected for by evolution. It could be that the granulocytes, which are the cells with the highest turnover in the hematopoietic system, are more sensitive to disturbance than other cells to i.e. cell cycle interruption and undergo apoptosis when their cell cycle is blocked by IFN I. It could also be that the virus follows an early overactivation strategy that results in eventual immunosuppression. In the model of IFN I receptor knockout mice that we used for superinfection, the mice did not show granulocyte apoptosis and overcame the superinfection with L.m. much better than wildtype mice. Therefore, absence of IFN I signalling is an advantage in this situation. However, these mice do no clear the virus and become carriers of noncytopathic viruses. In addition, when infected with a cytopathic virus such as VSV, they die within 2 days. Therefore, a functional IFN I signalling is of benefit. However, viruses could use this negative side-effect on hematopoiesis to induce temporary immunosuppression for establishing infections.

Granulocyte suppression could be common during virus infections

We believe that it could be of great benefit to measure some parameters of granulocyte activation and apoptosis in patients that are immunosuppressed. This could be done in virus infections where superinfections are regularly seen, i.e. in HIV

infection where also bone marrow suppression has often been observed and has not been explained as yet. Also, it would be interesting to analyse bone marrow samples of early influenza and measles patients - however, because of ethical reasons, this is difficult. The granulocyte count in the blood during influenza infection has been observed to be increased (Douglas, Alford et al. 1966) or lowered (Lewis, Gilbert et al. 1986). However, we believe the timepoint of measuring the granulocyte count is probably crucial. In the more conventional viewpoint of adaptive immunology, the short term kinetics can often be omitted to some extent because many effects are long-lasting. Indeed, also in LCMV infection, a bone marrow granulocytosis results eventually, but the window of opportunity that enhances superinfections comes early after infection.

Circulating bacterial products induce immunosuppression

Undue granulocyte activation can have detrimental effects not only during virus infection, but also during circulation of bacterial products. We observed that after injection of Pam2Cys, a synthetic ligand derived from a cell membrane component of mycoplasma, the bone marrow granulocytes were reduced by 95%. It has been observed long ago that circulation of bacterial products is detrimental for the immune response. Lipopolysaccharide leads to systemic overactivation that involves high secretion of TNF-α, hypercirculation and eventual blood pressure insufficiency and shock. Several clinical studies have been performed with anti-LPS antibodies (McCloskey, Straube et al. 1994) or anti-TNF antibodies (Abraham, Wunderink et al. 1995) to antagonize these effects. However, no clear benefits for patients resulted; therefore these agents are not in regular use. We have not tried anti-Pam2Cys antibodies or anti-TLR2 antibodies in our model and presume that these could have beneficial effects, but only because we stimulate only TLR2.

It should be kept in mind that a regular bacterial infection has probably several hundred different pattern recognition receptor activating molecules – therefore to give only anti-LPS during sepsis seems a too narrow an approach. In addition, a systemic cytokine storm involves many different cytokines that can lead to detrimental outcome. In our model, we have investigated whether the bone marrow suppression with Pam2Cys is mediated by TNF-α signalling or IFN I or II. However, bone marrow granulocytes were suppressed in TNF-Receptor 1 knockout mice after Pam2Cys treatment just as in wildtype mice. The same was true for IFN I Receptor and IFN II knockout mice. Therefore, bone marrow suppression by Pam2Cys is not due to one

of these cytokines alone. Even if a single cytokine could be identified to be mainly responsible for detrimental effects, both anti-IFN I treatment and anti-TNF or -LPS treatments are probably double-edged and very difficult to handle: Too much anti-IFN I could help virus persistence, too much anti-TNF lower antibacterial responses.

G-CSF is upregulated during bone marrow granulocyte suppression

During sepsis, also GM-CSF treatments have been tried in clinical studies. However, this was found to have no beneficial effect (Root, Lodato et al. 2003). In our models of granulocyte suppression, we have never observed a lack of G-CSF, the main cytokine responsible for granulopoiesis – in fact, as soon as some granulocyte loss occurred, G-CSF was upregulated rapidly. This could mean that additional GM-CSF / G-CSF administration during sepsis is futile and granulocyte numbers need to be preserved by other means.

Lasting granulocyte-dependent resistance

When we activated granulocytes and waited for 7 days, we observed that the numbers in the bone marrow were strongly increased after being suppressed early on (Chapter 9). Not surprisingly, at this timepoint a bacterial infection was cleared more effectively than in the non-stimulated state. The interesting aspect of this experiment is that 7 day long changes can be produced in the innate immune system by a single stimulation. That this could have been expected is obvious, but so far has never been analysed further – instead, innate stimuli are usually given only as adjuvants to induce some adaptive immune response, and the response by phagocytes are no matter neglected. However, we believe that this mechanism could have some important implications. For example, as the numbers of all granulocytes are increased, it could be expected that the response against most bacteria who are cleared by granulocytes are ameliorated. Therefore, if there were a way to induce a therapeutic granulocytosis, this would be of great benefit i.e. before visceral surgery, in patients in danger of nosocomial infections.

Blood and bone marrow granulocyte numbers are not always congruent

Great variability was observed when the relationship between granulocyte numbers in the blood and in the bone marrow was analysed. On one hand, we found that during bone marrow granulocytopenia, there were still some granulocytes left in the blood. On the other hand, granulocyte numbers did not de- or increase dramatically in the blood during periods when the bone marrow was depleted of granulocytes and they were found apoptotic in the spleen. Unfortunately, this represents a difficulty for

human studies, as for the analysis of these mechanisms, one would have to resort to bone marrow biopsies.

Alterations of the factors influencing granulocyte transit could explain these inconsistencies. It is known that upregulation of integrins on endothelium and chemotactic signals from inflamed tissues can strongly influence granulocyte migration (Cinamon, Shinder et al. 2004). Possibly, these signals can be so highly expressed that the half-life of granulocyte shortens, and high numbers of granulocytes leaving the bone marrow can stay undetected as during the infection with L.m.

No single chemokine or receptor responsible for granulocyte homing

It would also be very interesting to know which molecules are responsible for chemotaxis of granulocytes to sites of infection. It is known that chemokines are partly responsible, but no knockout approach of any molecule has so far yielded an absence of granulocytic infiltration. The ß2-integrin CD11b measured here as an activation marker has a role in granulocytic adherence to endothelium. However, it can be safely deleted without impairing granulocyte homing. Instead, as could be expected, granulocyte activation, phagocytosis, and attachment to glass surfaces is impaired. Consistent with the observation that activation of granulocytes induces apoptosis, CD11b knockout granulocytes demonstrate decreased activation and decreased apoptosis.

Taken together, analysis of granulocyte kinetics in man could yield new insights to better understand superinfection and sepsis. If inappropriate activation of granulocytes could be attenuated, the infections with pathogens where granulocytes play a significant role could be overcome more easily, and this may extend to bacteria resistant to antibiotics.

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11. Curriculum vitae

Personal Data:

Name:	Navarini Alexander Andreas
Date of birth:	Februar 14th, 1976 in Basel
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Schooling:

1983-1989	Primary school, Oberrohrdorf AG
1989-1996	Kanton school, Chur, GR
1996-2002	Medical studies at University of Basel

Clinical experience:

1998-1999	Tutorial with Dr. med. C. Scheidegger, FMH Internal medicine and infectiology, Basel
1999-2000	Exchange studies at Université Paris VI, Pitié-Salpétrière 3 months infectious and tropical diseases with Prof. Bricaire 1 month pneumology 1 month neurology 1 month gynecology 2 months emergency medicine and ambulance shifts at SAMU de Paris 1 month ophthalmology
2000-2001	Fifth year of medical studies: „Wahlstudienjahr“ 4 months internal medicine, KS Aarau 3 months surgery, KS Chur

	2 months tropical medicine, Schweizerisches Tropeninstitut Basel
	2 months dermatology, USZ
	5 months experimental research in dermatology, USZ
2002	Federal exams, University of Basel
	American medical exams USMLE Step 1,2
2003	Employed as „SOS Arzt“, Zurich:
	Family doctor emergency shifts, Family doctor replacement, Team doctor hockey club Thurgau
2006	6 months resident internal medicine, Bruderholzspital, Basel
2007	9 months resident internal medicine, infectiology, tropical medicine at Schweizerisches Tropeninstitut in Tanzania, HIV cohort study

Research experience:

1999	3 months molecular biology at Institute for Microbiology, Basel, with Prof. Moroni and PD Klimkait
2001	5 months doctoral thesis at Department of Dermatology, USZ: "Expression of Decorin in Cutaneous T-Cell Lymphoma and its effects on proliferation and apoptosis"
2003	Postgraduate course for experimental medicine and biology
since 11/2003	MD-PhD program as resident in experimental immunology with Prof. Zinkernagel at the Institute for Experimental Immunology, USZ. PhD thesis directed by Prof. A. Aguzzi, Institute for Neuropathology, USZ.

Civil service:

1998	4 months consultancy at Freiplatzaktion Basel, an advisory service regarding asylum specialzed for Sri Lankan Refugees.

Skills / Interests:

Foreign languages:	English, French, Italian
Interests:	Piano, Ski / Snowboard, Swimming, Watersports, Traveling

Publications:

1. Navarini AA*, Recher M*, Karl S. Lang, Georgiev P, Meury S, Bergthaler A, Flatz L, Bille J, Landmann R, Odermatt B, Hengartner H, and Zinkernagel RM. *„ Increased susceptibility to bacterial super-infection as a consequence of innate antiviral responses“* **Proc Nat Acad Sci** 2006, Oct 11 (* = shared first author)
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   Nat Immunol. 2004 Sep;5(9):934-42. Epub 2004 Aug 8.

12. Additional projects (and their publication) pursued during my PhD period:

Exposure to a lethal virus infection during maternal antibody protection can act as vaccination

(manuscript in preparation)

Navarini AA, Krzyzowska M, Recher M, Lang KS, Verschoor A, Hengartner H, Niemialtowski M and RM Zinkernagel

Abstract

Maternal antibodies have a recognized important role in conferring protection to the newborn against viruses the mother has already encountered. We demonstrate here that mice repeatedly exposed to a lethal dose of vaccinia virus survive when protected by maternal antibodies and generate their own protection. When later in life a challenge with the cross-reactive ectromelia virus was performed, only the mice exposed to vaccinia virus as pups survived while unexposed mice died. When maternal protection is available, then encountering a deadly virus infection early in life mediates vaccination via attenuation if repeated, with a huge impact on survival
.

Contribution: AA Navarini performed all experiments except virus challenge of adult mice.

Absence of Flt3L increases resistance to primary listeria monocytogenes infection

(manuscript in preparation)

Navarini AA, Lang KS, Recher M, Odermatt B, Hengartner H and R.M. Zinkernagel

Abstract

Flt-3L is known to be centrally implicated in hematopoiesis of dendritic cells. Knockout mice for this cytokine harbor dramatically lower dendritic cell counts than wildtype mice, leading to decreased sentinel functions of dendritic cells. Treatment of wildtype mice with recombinant Flt3L has been shown to increase resistance to the gram-positive bacterium Listeria monocytogenes in secondary infection of adult mice, and during infection of newborns. However, increased dendritic cell counts have also been reported to impair primary anti-listerial responses. Here we demonstrate that Flt3L knockout mice harbor less interferon I producing dendritic cells, have lower amounts of type I interferon in serum upon Listeria monocytogenes infection and are more than a 100-fold more resistant than wildtype mice during primary challenge. When Flt3L-/- mice were treated with recombinant Flt3L, susceptibility could be partially restored.

Contribution: AA Navarini performed all experiments.

Complement 3 and 4 deficiency results in increased resistance to listeriosis

(Ongoing project)

Verschoor A*, **Navarini AA***, Hengartner H and R.M. Zinkernagel

Abstract

Complement deficiency has been identified as an immune defect that predisposes patients to purulent infections. Here we demonstrate that for the intracellular bacterium Listeria monocytogenes, complement 3 and 4 deficiency leads to increased clearance of bacteria after 3 days. This is associated with increased granulocyte numbers in complement deficient mice. During the whole period of infection, granulocytes were elevated in complement deficient mice compared to controls. Further, L.m. showed decreased homing to complement deficient liver macrophages. These macrophages have only low bactericidal function and constitute a reservoir for L.m. to propagate within. With administration of cobra venom factor, we could inhibit L.m. homing to macrophages and induce anti-listerial resistance comparable to C3-/- mice.

Contribution: AA Navarini for half of all experiments.

VSV infection fails to activate cerebral innate protection

(ongoing project)

Falsig J, **Navarini AA**, Zinkernagel RM and A. Aguzzi

Abstract

Vesicular stomatitis virus is neurotropic and cytopathic with an intracerebral LD50 of 1-5 pfu. We have asked whether the brain cells are unprotected because innate reactivity is lacking. We observed that live cerebral tissue sections treated with interferon type I could be protected against VSV infection. However, they did not produce a significant amount of IFN I when inoculated with VSV or when treated with irradiated VSV particles. When they were stimulated with Poly(I:C), cerebral slices produced IFN I and were protected against VSV infection. Depletion of microglia did not abrogate this effect, therefore also other cerebral cells are capable of mediating this protection. When pure astrocyte and neuronal cell cultures were treated with Poly(I:C), they could also develop some protective function against VSV.

Contribution: AA Navarini performed infection experiments, titer determination and ELISA.

Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling

J Clin Invest. 2006 Sep;116(9):2456-63.

Lang KS, Georgiev P, Recher M, **Navarini AA**, Bergthaler A, Heikenwalder M, Harris NL, Junt T, Odermatt B, Clavien PA, Pircher H, Akira S, Hengartner H, Zinkernagel RM.

Abstract

The liver is known to be a classical immunoprivileged site with a relatively high resistance against immune responses. Here we demonstrate that highly activated liver-specific effector $CD8^+$ T cells alone were not sufficient to trigger immune destruction of the liver in mice. Only additional innate immune signals orchestrated by TLR3 provoked liver damage. While TLR3 activation did not directly alter liver-specific $CD8^+$ T cell function, it induced IFN-α and TNF-α release. These cytokines generated expression of the chemokine CXCL9 in the liver, thereby enhancing $CD8^+$ T cell infiltration and liver disease in mice. Thus, nonspecific activation of innate immunity can drastically enhance susceptibility to immune destruction of a solid organ.

Contribution: AA Navarini performed one of the mouse experiments with administration of pertussis toxin to block chemokine signalling.

Cholestasis protects the liver from ischaemic injury and post-ischaemic inflammation in the mouse

Gut. 2006 Jun 8

Georgiev P, **Navarini AA**, Eloranta JJ, Lang KS, Kullak-Ublick GA, Nocito A, Dahm F, Jochum W, Graf R, Clavien PA.

Abstract

Cholestasis is associated with high morbidity and mortality rates in patients undergoing major liver surgery, but the responsible mechanisms remain elusive. Increased ischaemic liver injury and inflammation might contribute to the poor outcome. Common bile duct ligation (biliary obstruction with hyperbilirubinemia) or selective ligation of the left hepatic duct (biliary obstruction without hyperbilirubinemia) was performed in C57BL/6 mice prior to 1h of hepatic ischaemia and 1, 4 or 24h of reperfusion. Infection with the intracellular hepatic pathogen Listeria monocytogenes for 12 and 48h was used to study ischaemia-independent hepatic inflammation. Unexpectedly, cholestatic mice displayed significant protection from ischaemic liver injury as determined by transaminase release, histological liver injury, and neutrophil infiltration. In cholestatic mice, reduced injury correlated with a failure to activate NF-eB and TNF-α mRNA synthesis, two key mediators of postischaemic liver inflammation. After selective bile duct ligation, both the ligated and the non-ligated lobes displayed blocked activation of NF-eB as well as reduced induction of TNF-α mRNA synthesis and neutrophil infiltration. In contrast, infection with Listeria monocytogenes revealed comparable activation of NF-eB and hepatic recruitment of neutrophils 12h after infection. Cholestasis does not enhance but rather dramatically protect from ischaemic liver injury and inflammation. This effect is mediated by a systemic factor, but not bilirubin, and is associated with a preserved capacity to trigger an inflammatory response to other stimuli such as a bacterial pathogen.

Contribution: AA Navarini performed all experiments with Listeria monocytogenes infection.

Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation

Eur J Immunol. 2005 Mar;35(3):738-45.

Lang KS*, Recher M*, **Navarini AA***, Harris NL, Lohning M, Junt T, Probst HC, Hengartner H, Zinkernagel RM. (* shared first authors)

Abstract

Persistence is a hallmark of infection by viruses such as HIV, hepatitis B virus, hepatitis C virus and LCMV. In the case of LCMV, persistence may often be associated with exhaustion of CD8($^+$) T cells. We demonstrate here that persistent antigen suppressed IL-7Rα expression and this correlated with T cell exhaustion and reduced expression of the anti-apoptotic molecule B cell leukemia/lymphoma 2 (Bcl-2). In contrast, exposure to short-lived antigen only temporarily suppressed IL-7Rα expression, failed to induce T cell exhaustion, and primed T cells. Persistent antigen also suppressed IL-7Rα expression on primed T cells and this correlated with exhaustion of a previously stable primed T cell population. These findings suggest that antigen longevity regulates T cell fate.

Contribution: AA Navarini contributed the concept of the IL-7R downregulation and performed FACS analysis.

Requirement for neutralizing antibodies to control bone marrow transplantation-associated persistent viral infection and to reduce immunopathology

J Immunol. 2005 Oct 15;175(8):5524-31.

Lang KS*, Recher M*, **Navarini AA***, Freigang S, Harris NL, van den Broek M, Odermatt B, Hengartner H, Zinkernagel RM. (* shared first authors)

Abstract

Bone marrow transplantation (BMT) is commonly used in the treatment of leukemia, however its therapeutic application is partly limited by the high incidence of associated opportunistic infections. We modeled this clinical situation by infecting mice that underwent BMT with lymphocytic choriomeningitis virus (LCMV) and investigated the potential of immunotherapeutic strategies to counter such infections. All mice that received BMT survived LCMV infection and developed a virus carrier status. Immunotherapy by adoptive transfer of naive splenocytes protected against low (200 PFU), but not high (2 x 10(6) PFU), doses of LCMV. Attempts to control infection of high viral titers using strongly elevated frequencies of activated LCMV-specific T cells failed to control virus and resulted in immunopathology and death. In contrast, virus neutralizing Abs combined with naive splenocytes were able to efficiently control high-dose LCMV infection without associated side effects. Thus, cell transfer combined with neutralizing Abs represented the most effective means of controlling BMT-associated opportunistic viral infection in our in vivo model. These data underscore the in vivo efficacy and immunopathological "safety" of neutralizing antibodies.

Contribution: AA Navarini performed the experiments with transfer of hyperimmune serum to prevent immunopathology (Fig. 7).

Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease

Nat Med. 2005 Feb;11(2):138-45.

Lang KS, Recher M, Junt T, **Navarini AA**, Harris NL, Freigang S, Odermatt B, Conrad C, Ittner LM, Bauer S, Luther SA, Uematsu S, Akira S, Hengartner H, Zinkernagel RM.

Abstract

Autoimmune diabetes mellitus in humans is characterized by immunological destruction of pancreatic beta islet cells. We investigated the circumstances under which CD8($^{+}$) T cells specific for pancreatic beta-islet antigens induce disease in mice expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) as a transgene under the control of the rat insulin promoter. In contrast to infection with LCMV, immunization with LCMV-GP derived peptide did not induce autoimmune diabetes despite large numbers of autoreactive cytotoxic T cells. Only subsequent treatment with Toll-like receptor ligands elicited overt autoimmune disease. This difference was critically regulated by the peripheral target organ itself, which upregulated class I major histocompatibility complex (MHC) in response to systemic Toll-like receptor-triggered interferon-α production. These data identify the 'inflammatory status' of the target organ as a separate and limiting factor determining the development of autoimmune disease.

Contribution: AA Navarini performed the experiments with IFNAR-/- x RIP-GP and LCMV infection (Fig. 6).

Deliberate removal of T cell help improves virus-neutralizing antibody production.

Nat Immunol. 2004 Sep;5(9):934-42.

Recher M, Lang KS, Hunziker L, Freigang S, Eschli B, Harris NL, **Navarini A**, Senn BM, Fink K, Lotscher M, Hangartner L, Zellweger R, Hersberger M, Theocharides A, Hengartner H, Zinkernagel RM.

Abstract

The B cell response to lymphocytic choriomeningitis virus is characterized by a CD4($^+$) T cell-dependent polyclonal hypergammaglobulinemia and delayed formation of virus-specific neutralizing antibodies. Here we provide evidence that, paradoxically, because of polyclonal B cell activation, virus-specific T cell help impairs the induction of neutralizing antibody responses. Experimental reduction in CD4($^+$) T cell help in vivo resulted in potent neutralizing antibody responses without impairment of CD8($^+$) T cell activity. These unexpected consequences of polyclonal B cell activation may affect vaccine strategies and the treatment of clinically relevant chronic bacterial, parasitic and viral infections in which hypergammaglobulinemia is regularly found.

Contribution: AA Navarini helped with several of the experiments.